THE ROLE OF ANTIBACTERIAL AGENTS ON THE PATHOGENICITY OF *STAPHYLOCOCCUS AUREUS*

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The difficulty in most scientific work lies in framing
the questions rather than finding the answers

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ABSTRACT

Staphylococcus aureus is a pathogenic organism which has an impressive armoury of extracellular toxic factors. In recent years, *S. aureus* has developed resistance to some of the new 4-quinolone antibacterials. Since this resistance is a result of a chromosomal mutation, and not plasmid mediated, it was proposed that the resistant bacteria might be less pathogenic. This thesis has studied this postulate.

Ciprofloxacin sensitive and resistant strains of *S. aureus* were compared to establish any difference in their respective production of virulence factors both *in vitro* and *in vivo*. Since *S. aureus* produces a large number of virulence factors, four were studied in detail; coagulase, protein A , α and δ haemolysin.

The *in vitro* production of the four factors by ciprofloxacin sensitive and resistant strains was investigated in the presence and absence of 1/4 and 1/2 minimum inhibitory concentration (MIC) levels of six antibiotics: ciprofloxacin, enoxacin, methicillin, gentamicin, chloramphenicol and tetracycline. The production of soluble and bound coagulase was measured by a chromogenic substrate assay. The production of cell bound and extracellular protein A, α and δ haemolysin was measured by ELISAs. In the absence of the antibiotics, the production of the four virulence factors was similar in the resistant

and sensitive strains. The acquisition of ciprofloxacin resistance did not affect the production of the factors. Conversely, the presence of 1/4 and 1/2 MIC levels of gentamicin, chloramphenicol and tetracycline reduced the production of both bound and soluble coagulase, and protein A, in both sensitive and resistant bacteria. In the presence of 1/2 MIC levels of ciprofloxacin, enoxacin, chloramphenicol and tetracycline the production of α and δ haemolysin was reduced and in some cases completely inhibited. The presence of 1/4 MIC levels of the antibiotics had a reduced effect on the inhibition of the toxins. The presence of gentamicin at 1/2 or 1/4 MIC levels had little effect on haemolysin production. The presence of 1/4 and 1/2 MIC levels of methicillin both increased the production of the toxins, but the increase was less significant in the presence of 1/4 MIC levels.

In order to correlate these *in vitro* results with pathogenicity, an *S. aureus* abscess model in mice was used, since this allowed easy comparisons of strains under *in vivo* conditions. Paired strains of ciprofloxacin sensitive and resistant bacteria were injected subcutaneously into mice and the resulting lesions examined. The ciprofloxacin phenotype did not affect lesion production or degree of tissue damage. The effect of 1/2 and 1/4 MIC levels of ciprofloxacin on the development of the abscesses was also investigated, and it was shown that they reduced the size of the abscesses if treatment was started one hour after the subcutaneous injection of *S. aureus*. The number of

viable organisms recovered from mice treated with sub MIC levels of ciprofloxacin was less than the number recovered from control mice. Ciprofloxacin resistant bacteria were recovered from abscesses of mice treated with the antibiotic for 24 hours.

Contents

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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Publications

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S Doss, GS Tillotson, SGB Amyes (1991) Effect of 4-quinolones on the production of pathogenicity factors in staphylococci. Book of Abstracts, 5th European Congress on Clinical Microbiology and Infectious Disease, Oslo, Norway, Abstract no. 1413.

1 Introduction

In general terms, the type of association between two different organisms can be listed as parasitic, where one benefits and one doesn't, or symbiotic, where both benefit. There is an intermediate category, where only one organism derives benefit without doing damage to the other organism and this is termed commensalism.

The association between man and microorganisms could be classified as symbiotic - bacteria in the gut providing vitamins, or as commensal - bacteria on the skin. Only a small number of microorganisms associated with man give rise to pathological changes or cause disease. This is not a surprising revelation since successful parasitic microbes need to maintain themselves in nature and avoid extinction, i.e. live on or in the host, multiply and then spread to a new host. The parasitic microbe will try to get as much as it can from the host without causing too much damage, but some degree of tissue damage may be necessary. Different microbes have attained different balances in this respect.

The hosts have developed a highly efficient recognition system for invaders, and inflammatory and immune responses to restrain their growth and spread, and eliminate them from the body. The microorganisms, for their part, have evolved and developed ways of bypassing the host defences. The ability to adapt and evolve, and exploit

weak points in the host defences, is a trait shared by most successful microorganisms.

This thesis is going to examine one particular microorganism *Staphylococcus aureus* which has been described as a successful pathogen (Mudd 1970). Its pathogenic determinants will be described, as will their importance in causing various diseases and how they can be "attenuated" by antimicrobials. First it is necessary to look at the various host defences and the general principles of microbial pathogenicity.

1.1 Host Defences

It is convenient to divide host defence mechanisms against infection into those that operate externally and those that function internally.

1.1.1 External Defences

The skin presents a mechanical barrier to penetration of microorganisms and infection of the skin usually takes place only when the continuity of the skin has been mechanically broken.

The skin possesses an indigenous microflora (Price 1938), which is mostly non pathogenic (Williams 1973). It usually has an acid pH (Noble 1968) and long chain fatty acids, which are bactericidal, occur in sebaceous gland secretions (Pillsbury and Rebell 1952; Aly *et al* 1972).

The conjunctiva and the alimentary, respiratory and urogenital tracts offer pathways for infection by microorganisms. The cornea is covered by lacrimal fluid (tears) which contains lysozyme (Fleming 1922), an enzyme that has an antibacterial function (Zinder *et al* 1956). The respiratory tract, particularly the upper respiratory tract is exposed to a wide variety of particulate material, including microorganisms. The majority of the particles are trapped by the secretions that cover the mucous membranes (McNabb and Tomasi 1981) and once trapped, the particles are transported by means of a mucous stream to the base of the tongue, where they are periodically swallowed (Richardson 1987). The epithelial surfaces of the gastrointestinal tract are covered by a layer of mucus. It contains components such as lysozyme which attacks susceptible bacteria (Creeth *et al* 1979) and lactoferrin which may deprive bacteria of iron, an essential growth requirement (Emery 1980). In the urinary tract mechanical elimination is the most important factor (Mooney *et al* 1976; O'Grady *et al* 1988).

1.1.2 Internal Defences

The internal defence mechanisms can be divided broadly into non specific immunity and specific immunity. Non specific immunity can be described as those processes which, without being directed against any specific microbe, help to prevent infection. Specific immunity involves some degree of special recognition and sometimes the immunity may not extend beyond a particular bacterium.

Non Specific Immunity

Non specific factors are soluble materials present in the tissues and body fluids such as blood lymph, tears, saliva and mucus. Lysozyme, found in many body fluids, splits cell wall peptide (Muschel 1963) and beta-lysins, whose amounts increase during infection lyse Gram positive bacteria (Myrvik and Leake 1960). Transferrin and lactoferrin present in serum, milk and other secretions, prevent the growth of many pathogens by denying them essential iron (Weinberg 1971).

If bacteria penetrate into or through epithelial surfaces, they act as foreign bodies and therefore stimulate an inflammatory response. The most important event is the accumulation of large numbers of phagocytic cells at the inflammatory site. Although these cells also function to remove microbial and host tissue debris and to dispose of inflammation-producing factors, they are particularly important in engulfing and destroying pathogenic bacteria (Ryan and Majno 1977). An early inflammatory response has been shown to be a major protective factor against *Staphylococcus aureus* infection (Easmon and Glynn 1975a).

Specific immunity

Specific immunity involves phagocytosis and antibodies. Phagocytosis is the ingestion of invading bacteria into the cytoplasm of cells (Hirsch 1974). Phagocytosis is greatly improved by the presence of antibodies

which the body has produced as a result of such contact. The process whereby substances improve phagocytosis is called opsonisation (Wright and Douglas 1903). Protein A , a cell wall component of *S. aureus* interfers with opsonization and phagocytosis (Peterson *et al* 1977). Antibodies are proteins produced as a result of the introduction of substances which the body recognises as foreign (antigens). They are supported by the complement system. Complement, a normal constituent of human blood plasma (Muller-Eberhard 1969), can be subdivided into two pathways; the classical pathway activated by IgG and IgM-type complexes (Muller -Eberhard 1969) and the alternative pathway activated by aggregates of IgA and naturally occurring polysaccharides and lipopolysaccharides (Muller-Eberhard 1971). They act to produce a cascade of enzyme reactions culminating in the activation of a molecular complex which will attack and dissolve the phospholipid wall of bacteria and tissue cells (Kolb *et al* 1972; Poddack and Tschopp 1982).

1.2 Microbial Pathogenicity

Microbial pathogenicity has been defined as "the biochemical mechanisms whereby microorganisms cause disease" (Smith 1968). Not all pathogens have the same capacity to cause infection and disease. Microbial pathogenesis is usually complex and multifactorial. Pathogens have several biochemical mechanisms which may act individually or together to produce disease. Removal of any one of these components

may or may not render the organism avirulent. To be pathogenic a microorganism must be able to: (1) Break through the skin barrier (by trauma or insect bite) or infect the mucous surfaces, (2) Enter the host -penetration of the mucous surfaces, (3) Multiply, (4) Resist or interfere with host defence mechanisms, and (5) Cause damage to the tissue of the host (Smith 1984).

1.2.1 Break through skin / Infect mucous surface

Large or small breaks in the skin resulting from wounds are obvious routes for infection. Bites are also important sites for the entry of microorganisms.

The majority of infections start on the mucous surface. When a pathogen enters the alimentary, respiratory or urogenital tract, the first requirement for infection is to make contact with the epithelial surface through the overlaying mucus layer (Freter 1980). After contact is made the pathogen must adhere to the surface, to prevent removal by moving lumen contents or mucociliary action.

Factors produced by *Haemophilus influenzae* seem to result in the immediate slowing of ciliary beating. This would have the effect of delaying mucociliary clearance and giving the bacterium time to multiply and bind with the receptors on epithelial cells (Wilson *et al* 1986).

Attachment is mediated by cell-surface structures termed "adhesins" (Duguid 1959). Some strains of *Escherichia coli* which cause diarrhoea produce surface antigens and colonisation factor antigens, which are responsible for the adherence of the bacteria to the surface of the intestine (Beachey 1981). Other bacterial surface components contributing to their adherence to mucous membranes include carbohydrates, glycoproteins, glycolipids, K antigens, M proteins and pili (Lancefield 1962; Smith 1977; Arbuthnott and Smyth 1979).

Fibronectin is a large multifactorial, extracellular matrix and plasma glycoprotein which promotes adherence functions in mammalian cells. This molecule also adheres in large quantities to mucosal surfaces. *S. aureus* and *Streptococcus pyogenes* adhere to fibronectin on epithelial cell surfaces (Beachey and Courtney 1987; Proctor 1987). Although these two organisms adhere to the same molecule under similar conditions, they use different mechanisms (Mosher and Proctor 1980; Courtney *et al* 1983).

To maintain infection on mucous surfaces pathogens must be able to resist the host defence mechanisms located there, such as acid/alkaline pH, bactericidal materials and extruded phagocytes. They must be able to compete with their neighbours for space on the host surface and possibly for nutrients (Savage 1972). Bacteria overcome these mucosal defences by devices similar to those used later within the tissues.

1.2.2 Entry to the host

Upon attachment, one of these three disease producing processes occur:-

(1) The parasite may multiply upon the surface of the epithelial cell without penetrating into the cell or deeper tissue. Disease in this instance is caused by the elaboration by the parasite of a soluble toxin substance that is absorbed through the mucous membrane, producing local or distant tissue damage e.g. in the disease diphtheria - the causative agent *Corynebacterium diphtheriae* attaches to the epithelium of the pharynx, where it elaborates a potent toxin that is absorbed and transported to other tissues. All symptoms of the disease can be attributed to activities of this toxin (Pappenheimer 1965; Bowman and Bonventre 1970).

(2) The parasitic bacteria may attach to epithelial cells, then penetrate into these cells where they multiply and produce disease by destroying the epithelial cell layer. An example of this is *Shigella flexneri*, which produces dysentery without penetrating into submucosal tissues. The mechanism by which bacteria of this type penetrate into epithelial cells is not well understood but they seem to enter by a process resembling phagocytosis. The infection is usually confined to the superficial layers of the intestinal mucosa, and the organisms spread to the other surface epithelial cells and cause much tissue damage, excessive fluid secretion and inflammation (Formal *et al* 1983; Hale and Formal 1987).

(3) Certain pathogenic bacteria, after attachment to epithelial cells may enter these cells and pass through them into submucosal tissues, often

without significant damage to the mucosal cells. The factors and forces involved in this phagocytosis and transport of the parasite through epithelial cells are not known (Takeuchi and Sprinz 1967). Invasion of the gastrointestinal tract is an essential step required for *Salmonella typhimurium* pathogenesis (Giannella *et al* 1973). Most *Salmonella* species proceed through the surface intestinal epithelial cells into deeper tissue and often enter reticuloendothelial cells. They are ingested by macrophages, within which they multiply readily.

1.2.3 Bacterial Multiplication

In order to produce disease, a pathogenic bacterium, once it has escaped the external defence mechanisms and gained access to sub-epithelial tissue, must multiply, since the number of pathogenic bacteria that infect the host is too small to produce disease immediately.

Only in a few cases and early in infection has the multiplication rate *in vivo* been measured (Smith 1983). This rate was much slower than that seen in laboratory cultures, suggesting that limiting nutritional and physiological conditions may exist *in vivo* before tissue breakdown occurs. The nutrients or other environmental factors that limit growth at the beginning of infection are largely unknown except for the influence of iron supply. Most bacteria need iron to grow (Weinberg 1966) and *in vivo* the amount of free iron is restricted by iron-complexing host proteins, transferrin and lactoferrin. Virulent

strains secrete siderophores into the surrounding environment and so obtain sufficient iron (Griffiths 1983).

1.2.4 Resist or interfere with host defences

At the beginning of any infection, the non specific defence mechanisms act to prevent or reduce it. If infection occurs this is strengthened by specific immune response which usually eliminates the infection. Therefore, to make progress against the host defences, a pathogenic bacterium must be able to inhibit or interfere with the humoral factors, the phagocytes, complement and the immune response.

Interference with humoral factors

The major humoral bactericidins are the later components of the complement cascade for Gram negative bacteria and lysozyme, beta lysins and basic peptides for Gram positive bacteria (Smith 1976; Penn 1983). Resistance to humoral bactericidins has been shown by virulent strains *Bacillus anthracis* (Keppie *et al* 1963), *S. aureus* (Cybulska and Jeljaszewicz 1966) and some *Enterobacteriaceae* (Braun *et al* 1960).

Interference with the action of phagocytes

The four stages of the phagocytic defence system are: (1) mobilisation by inflammation (2) chemotaxis towards bacteria (3) attachment and engulfment (4) killing by oxygen dependent systems.

Mobilisation may be prevented by an active process e.g. virulent staphylococci produce an anti inflammatory cell wall peptidoglycan (DOCR; deoxycholate residue) (Easmon *et al* 1973). They also produce leukocidins which dispatch phagocytes which are attracted to sources of inflammation (Rogers and Tomsett 1952).

Chemotaxis was prevented *in vitro* by fractions from tubercle bacilli (Dubos and Hirsch 1965) and a cell wall product of staphylococci which was different from the anti inflammatory mucopeptide (Weksler and Hill 1969; Glynn 1972).

Resistance to attachment and ingestion by phagocytes of virulent strains was one of the first areas of bacterial pathogenicity to receive attention. In most cases inhibition of ingestion has not been distinguished from inhibition of attachment. Bacterial surface components (aggressins) can interfere with ingestion of invading microorganisms by phagocytic cells (Glynn 1972; Smith 1977). There are two general types of aggressins: surface and capsular products which do not appear to harm phagocytes, and toxic materials that do direct damage. These antiphagocytic factors include the polysaccharides of meningococci and pneumococci (Dubos and Hirsch 1965), the M protein and hyaluronic acid of streptococci (Fox 1974), protein A and coagulase of staphylococci (Peterson *et al* 1977b). These capsules are weakly immunogenic and mask more immunogenic underlying bacterial surface structures and would directly activate

complement. Toxic aggressins probably harm phagocytes in the same way as they affect ordinary cells; for example interference with membrane function by α toxin of *S. aureus* (Bhakdi *et al* 1989).

Interference with the action of complement

Mobilisation of phagocytes and their chemotaxis towards bacteria are promoted by earlier components of the complement cascade which also opsonise the bacteria for attachment and ingestion by phagocytes (Wilton 1981; Penn 1983). Preventing activation of complement or destroying its components can interfere with both humoral and cellular defences. Some surface or capsular materials of bacteria have been strongly implicated in masking cell-wall components which would otherwise activate complement directly or after reaction with natural antibody. Capsular polysaccharides of *Streptococcus pneumoniae* are often poor activators of the alternative and classical pathways in the absence of specific antibody (Giebink *et al* 1978). Some bacteria destroy components of complement thereby preventing its action. *S. aureus* produces and sheds a decomplementation antigen (Bhakdi and Muhly 1985) which consumes early but not terminal pathway components from serum in conjunction with antibody. Many Gram positive bacteria are innately resistant to lysis by complement, which might be explained by the cell wall acting as a barrier hindering access of complement to the cytoplasmic membrane (Braude 1981).

Proteus and *Klebsiella* spp inactivate complement. Their urease splits urea in the kidney and thereby releases ammonia, which can inactivate complement (Braude 1981). The elastase of *Pseudomonas aeruginosa* destroys part of the complement cascade (Schultz and Miller 1974; Densen and Mandell 1980).

Interference with the immune response

Bacteria can interfere with the immune response either by directly suppressing it or by subverting its action. Many bacteria, particularly those causing chronic infections, suppress the action of B and T cells by various mechanisms (Falconi and Campa 1981).

Bacteria can also subvert the immune response by one of three ways. First, the determinants can be poor antigens, i.e. they stimulate the immune response inadequately or nor at all. Thus, the determinants will not be completely neutralised by the immune response and infection will persist (Malakian and Schwab 1967; Finger *et al* 1971). Second, bacteria can undergo antigenic change or shift; when the host has responded immunologically to their surface antigens, new bacterial types emerge with different surface antigens and the immune response is rendered ineffective. This shift seems to occur in some bacterial disease and during persistence of potentially pathogenic commensals on mucous surfaces. The surface antigens of *Borrelia recurrentis* seem to change during relapsing fever (Smith 1984). Finally, the bacteria may

hide away in cells such as epithelial cells or impaired mononuclear phagocytes; within these cells the bacteria are protected against the host defence mechanisms. Examples of bacteria that hide in cells are *Salmonella* spp (Roantree 1967), *Shigella flexneri* (Yee and Buffenmyer 1970) and *Mycobacterium tuberculosis* (D'Arcy Hart and Armstrong 1974).

1.2.5 Damage to the host

Pathogenic bacteria damage the host tissues in two ways: production of toxins and stimulation of harmful immunological reactions.

Production of toxins

Most pathogenic bacteria form toxins, which are responsible for the harmful, sometimes lethal effects of disease. Most toxins are extracellular and they can inhibit protein synthesis, produce fluid loss from the gut, interfere with nerve action, lyse cells and cause vascular effects. Toxin elaboration may also inhibit the immune response of the host or perhaps in the case of enterotoxins, flush away competing bacterial neighbours (Cantey 1985). The most important cell-bound toxins are endotoxins in the cell walls of Gram negative bacteria, these are lipopolysaccharides whose toxicity appears to lie in their lipid portion (Stephen and Pietrowski 1981).

Toxins are often just one of several virulence factors produced by

pathogens, and although toxins may represent the principal determinant of virulence, and the cause of disease, they may not be the principal determinant of infectivity.

Damage by immunopathological mechanisms

Bacteria can produce severe, even fatal effects on the host by stimulating an immune response to otherwise non-toxic bacterial components, and then interacting with this response in a subsequent infection or late in a chronic infection in a manner which damages tissue immunopathology. There are four types of immunopathological reaction: immediate hypersensitivity, Arthus type responses, cytotoxic reactions and delayed hypersensitivity (Parish 1972). Immediate hypersensitivity is where IgE antibody is present on mast cells and releases histamines, thereby producing vascular and respiratory effects seen in hay fever or asthma. Arthus type reactions occur when antigen-antibody complexes are deposited on tissue, which then gets attacked by phagocytes and causes tissue damage. This seems to happen in kidney damage following infections with *Proteus mirabilis* and streptococci (Parish 1972). Delayed hypersensitivity reactions occur where cell mediated immunity stimulated by previous interaction with the bacteria, mobilise mononuclear phagocytes to the site of additional or persistent infection and cause tissue damage (Rich 1951; Easmon and Glynn 1975b).

1.3 *Staphylococcus aureus*

As already mentioned, a successful parasite must gain access to its host, must survive and multiply, then leave the host and spread to a new host. *S. aureus* is a pathogenic bacterium *par excellence* which has become adapted to the fulfilment of these requirements. They are among the most important bacteria causing disease in humans. They are normal inhabitants of the human upper respiratory tract, skin, intestinal tract and vagina and are particularly prone to produce infection when a foreign body has lowered host resistance. These microorganisms also are noteworthy for the production of large numbers of exotoxins and other substances, some of which undoubtedly play an important role in their capacity to cause disease (Arvidson 1983).

The name Staphylococcus is derived from the Greek word Staphule, meaning cluster of grapes (Ogston 1882). These cells are able to divide on more than one plane, and so as a result the cells form irregular masses resembling bunches of grapes (Baird-Parker 1972).

1.3.1 Pathogenesis

Staphylococci may produce disease in almost every organ and tissue of the body. The skin is particularly prone to infection and it is thought that staphylococcal skin infections are probably the most common of all infectious diseases. *S. aureus* infections may spread by extension to contiguous tissues or by way of lymphatics and then blood. The disease

process is initially localised wherever *S. aureus* lesions occur. There is an acute inflammatory response and an accumulation of enormous numbers of segregated neutrophils. Lesions tend to be walled off, owing to the subsequent deposition of fibrin, central necrosis appears and an abscess is formed. Thus staphylococcal infections are characterised by abscess formation. Dissemination may occur from an initial abscess.

It is not only *S. aureus* which is pathogenic, since coagulase negative staphylococci have become formidable nosocomial pathogens (Horan *et al* 1986). There is evidence that coagulase negative staphylococci produce a number of virulence factors that resemble those of *S. aureus* (Gemmell and Roberts 1974). They have the ability to adhere irreversibly to and grow on polymer surfaces and to produce an extracellular slime substance (Christensen *et al* 1985).

1.3.2 Virulence factors

Probably no other bacterium produces as many extracellular toxins, haemolysins, enzymes and cellular components, all of which at one time or another have been thought to be responsible for the virulence of *S. aureus*.

It is not possible to state that any one of a combination of these factors accounts for the virulence of *S. aureus*. Probably many of them play some role in the pathogenesis of staphylococcal disease, since

pathogenicity is a multifactorial process (Smith 1984). As previously mentioned, five requirements have to be fulfilled in order for disease to occur. These requirements can be broadly placed into two groups - entry and infection, and multiply and resist host defences. The pathogenicity factors of *S. aureus* can be placed in these two groups as well.

Table 1. Virulence factors of *Staphylococcus aureus*

Toxins	**Exoenzymes**	**Cell Bound Factors**
α	Coagulase	Clumping Factor
β	Staphylokinase	Protein A
γ	Proteases	Fibronectin binding protein
δ	Phospholipase	
Leucocidin	Lipase	
Exfoliative toxin	DNase	
Toxic Shock Syndrome Toxin-1	Phosphatase	
	Hyaluronidase	
Enterotoxins (SEA-SEE)		
Pyrogenic exotoxin		

Entry and infection of mucosal surface

S. aureus is particularly well endowed to colonise the outer or inner surface of its host, or to survive in sites of injury or infection. Resistance to desiccation and to skin fatty acids might be important in the early stages of infection. Methicillin-resistant *S.aureus* isolated from a burns unit, where spread was through the air, environment or staff contacts were significantly more resistant to desiccation than were strains common on a neonatal unit where hand-borne transmission is predominant (Farrington *et al* 1992). All staphylococci possess lipase and

esterase, which are able to breakdown the fats and waxes of the skin (Stewart 1965). *S. aureus* is also able to transport and oxidise amino acids, and metabolise carbohydrates (Ivler 1965).

Capsulation was found to provide increased protection from bactericidal lipids (Mortensen and Kapral 1992). However the effect was limited with only a 3-4 fold greater resistance in the capsulated strains than in the non-capsulate strains (Mortensen and Kapral 1992). Also, a strain which was very resistant to the bactericidal lipids was not capsulate, so other mechanisms besides capsule formation must be involved in sensitivity to lipids.

Pathogenic staphylococci are in practice distinguished from non-pathogenic strains by the secretion of coagulase (Cruiskshank 1937; Christie *et al* 1946). Coagulase is a protein which has the capacity to cause the clotting reaction of plasma (Zajdel *et al* 1976), by forming a stoichiometric, non-covalent complex with prothrombin (Hemker *et al* 1975). The formation of staphylothrombin does not involve proteolytic cleavage of the blood protein. It converts fibrinogen into insoluble fibrin in a manner similar to the action of physiologically formed thrombin (Hemker *et al* 1975; Zajdel *et al* 1976). The fibrin network may help form a wall round the staphylococcal infection site or, it may impede the final access of phagocytic cells, as well as depositing host fibrin on the bacterial cell wall, which therefore presents a less foreign surface to phagocytic

cells (Suter 1956; Jeljaszewicz *et al* 1983).

A second factor usually present at the surface of virulent staphylococci is bound coagulase (or clumping factor) (Duthie 1954; Engels and Kamps 1981). It is an antigenic protein which adsorbs fibrinogen with resulting aggregation of the staphylococcal cells. It is related antigenically to the extracellular Staphylocoagulase and is linked hydrophobically to the cell membrane (Engels and Kamps 1981). It has been suggested that it is an intermediate in the secretion process (Engels and Kamps 1981). It binds fibrinogen in the absence of prothrombin, which is the basis for the clumping reaction of staphylococci (Boden and Flock 1989). Clumped staphylococci are held together by fibrin fibres originating from the cell surface (Umeda *et al* 1980).

It has long been inferred that coagulase is a virulence factor and coagulase negative strains are less virulent than coagulase positive *S. aureus* (Kinsman and Arbuthnott 1980). This was supported by observations that coagulase-deficient mutants of *S. aureus* are less virulent for mice (Hasegawa and San Clemente 1978; Josson *et al* 1985). However, in these cases the mutants were isolated following chemical mutagenesis, so the loss of virulence may be due to mutation(s) which affect other factors, or the ability of the organisms to grow *in vivo* could have been compromised. A recent study reported the isolation of a single-site, coagulase deficient mutant by allele-replacement, which

would only affect the expression of coagulase. The coagulase negative mutant was no less virulent than otherwise isogenic coagulase positive strains both in terms of skin lesions and mastitis infections of mice (Phonimdaeng *et al* 1990).

The majority of coagulase positive staphylococci produce Staphylokinase which is fibrinolytic. Staphylokinase activates plasminogen to plasmin and is indirectly responsible for the fibrinolytic activity of *S. aureus* (Lewis and Ferguson 1951). They also produce hyaluronidase (spreading factor), an enzyme capable of hydrolysing the mucoid ground substance of the connective tissue (Linker *et al* 1956). The term 'spreading factor' originates from Duran-Reynals who found that extracts from S. *aureus* culture fluid enhanced the intracutaneous spreading of vaccinia virus, toxins and dyes (1933). It is not thought to be of clinical importance but it might have a role in the initial stages of infection (Elek 1959).

Fibronectin, which is a high molecular weight glycoprotein found in blood and other body fluids, has been shown to bind *S. aureus* (Kuusela 1978; Verbrugh *et al* 1981). The binding of fibronectin to *S. aureus* is mediated via the 27 kilodalton fragment of the amino terminus (Mosher and Proctor 1980). Fibronectin has been shown to increase adherence of bacteria to neutrophils (Verbrugh *et al* 1981; Proctor *et al* 1982b). Several groups have tried to isolate and purify the native cell wall associated

fibronectin protein of *S. aureus*, and there now seems to be full agreement that the fibronectin binding protein is 210 kilodalton as determined by SDS-PAGE (Froman *et al* 1987). The presence of fibronectin at sites of tissue damage may allow *S. aureus* to adhere. This is supported by the observation that a quantitative relationship exists between the number of fibronectin binding sites and the tendency of *S. aureus* to invade (Proctor *et al* 1982a). Besides serving as a substrate for bacterial attachment and colonisation, fibronectin has also been suggested as a factor in host defence. Fibronectin opsonizes *S. aureus* for neutrophils (Proctor *et al* 1979), which might be important for neutrophil activity in early *S. aureus* infection. Duckworth and Jordens (1990) found that methicillin resistant *S. aureus* (MRSA) adhere less well to HEp2 cells and fibronectin than methicillin-sensitive *S. aureus*.

Multiplication and resistance to host defences

S. aureus produces a large number of extracellular and cell bound toxic factors - at least 30 have been identified (Bernheimer and Schwartz 1961). Some of these will be considered individually.

Alpha toxin

Since the early studies of Burnet (1930) to a recent study by Bhakdi and Tranum-Jensen (1991), α toxin has been the most intensely studied staphylococcal toxin. Its main significance in pathogenicity is that of producing tissue damage after establishment of a focus of infection. It is

dermonecrotic, haemolytic to erythrocytes (Bernheimer 1965) and lethal to animals in sufficient dosage (Jeljaszewicz *et al* 1969). It produces spasm and paralysis of both smooth and skeletal muscle (Thal and Egner 1961) and can destroy or damage a wide variety of tissue culture cells, as well as leukocytes (Szmigielski *et al* 1967), platelets (Bernheimer and Schwartz 1965) and lysosomes (Bernheimer and Schwartz 1964).

The primary mechanism of membrane damage is the formation of pores (Fussle *et al* 1981). After binding to target membranes, toxin molecules oligomerize to form noncovalently associated, stable hexameric protein complexes. This process is associated with an exposure of lipid-binding domains, that enable the hexamer to insert spontaneously into the membrane. The hollow interior of the hexamer generates a hydrophilic channel across the lipid bilayer (Bloomquist and Thelestam 1988; Forti and Menestrina 1989). Toxin binds to target cells via two distinct types of interaction. At low concentrations, the toxin binds exclusively to a specific site, and at high concentrations, the toxin binds to membranes via a non specific absorptive interaction (Hildebrand *et al* 1991). The pores (1-2 nm diameter) permit free passage of ions and low-molecular weight molecules such as nucleotides, so cell death can be expected to ensue. Permeabilized erythrocytes undergo irreversible osmotic swelling, and haemoglobin is finally liberated as the membrane ruptures. Death of a particular cell type can have some primary biological consequences as a direct result of loss of the respective cell function. For example,

monocytes and macrophages are effectively killed by α toxin, and this conceivably cripples the phagocytic defence system in tissues (McGee *et al* 1983; Bhakdi *et al* 1989). It seems probable that such a powerful enzyme, producing injurious changes in the membrane permeability of a wide variety of cells, must have survival value to the parasitic staphylococcus in the tissues of its host. This is especially true in the light of *S. aureus'* ability to metabolise breakdown products of cells (Arvidson 1983).

Mutants of *S. aureus* deficient in α toxin production are less virulent than parental strains (Van der Vijer *et al* 1975; Jonsson *et al* 1985). These mutants were chemically induced and other characteristics might have been altered which also affect the virulence. Another major difficulty in these experiments, is that the production of several potentially important virulence factors in addition to α toxin are under regulation by a common control element (Yoshikawa *et al* 1974; Bjorklind and Arvidson 1980; Coleman 1981). The use of genetically engineered, isogenic bacterial strains differing solely in the expression of the α toxin gene gives proof for a pathogenic role of the toxin. In a study with isogenic strains α toxin was shown to be the major causative factor in the development of staphylococcal lesions in animal infections (Patel *et al* 1987). In another study employing site directed mutagenesis, an α toxin producing strain was more virulent than the toxin deficient isogenic strain in a mouse peritonitis model (O'Reilly *et al* 1986).

Beta toxin

This toxin is produced mainly by strains of *S. aureus* of animal origin, although human strains may produce it as well (Sperber 1977). It appears to be much less toxic than α toxin. It is a phospholipase C specific for sphingomyelin in the cell membrane (Wadstrom and Molby 1971; Molby 1976). The property which first focused on β toxin was the classic hot-cold haemolytic phenomenon (Bigger *et al* 1927). Little or no lysis occurs when β toxin is incubated at 37°C in the presence of Mg^{2+} with sheep erythrocytes. But if the reaction mixture is chilled for a period below 10°C, dramatic rapid lysis takes place. The sensitivity of erythrocytes of different species to β toxin is directly related to the relative amount of sphingomyelin in the membrane phospholipids. The most sensitive species are sheep, goat and ox, since their membrane phospholipids consist of about 50% sphingomyelin (Wiseman and Caird 1967). It has been shown that β haemolysin inhibits the Fc region of immunoglobulin from binding to monocytes, lymphocytes and polymorphonuclear leucocytes (PMNs), as well as being a negative chemotactic substance (Wilkinson 1977).

Gamma haemolysin

The production of a γ haemolysin from strains of staphylococci was demonstrated by Smith and Price (1938). It consists of two distinct cationic proteins (Taylor and Bernheimer 1974), which act synergistically. A recent report (Clyne *et al* 1992) suggests that the γ_1 component is a

protease or protease-like molecule that acts specifically on the γ_2 component to give enhanced haemolysis. The toxin exhibits a specificity for rabbit, human and sheep erythrocytes (Fackrell and Wiseman 1976). In this respect it resembles α toxin however it differs from it antigenically (Mollby 1983).

Delta toxin

δ toxin differs in many respects from the other three haemolysins excreted by staphylococci in its characteristics, properties and mechanisms of action, which is detergent like. It can damage a variety of cell types as a result of this activity (Rahal 1972; Wadstrom and Molby 1972). The toxin is lytic for erythrocytes and other mammalian cells, intracellular organelles and bacterial protoplasts and spheroplasts (Kreger and Bernheimer 1971). It has been shown to interact with many types of phospholipid monolayers as well as cod and sheep erythrocyte lipid films (Bhakoo *et al* 1982) suggesting an amphiphilic structure. The toxin forms an unusually stable monolayer and induces membrane permeability changes of large unilamelar vesicles composed of structurally defined lipids (Bhakoo *et al* 1985). It causes lesions in membranes strikingly similar to those caused by melittin and the detergent Triton X-100 (Thelestam and Mollby 1975). δ toxin is thought to be a surfactant because it is inhibited by phospholipids (Kreger 1970) and dilute normal serum (Jackson and Little 1958), has a low specific haemolytic activity and lyses a wide range of cell types (Kreger *et al* 1971).

It affects erythrocytes of all animal species without the significant differences in susceptibility so characteristic of α and β toxin (Kayser and Raynaud 1965).

Panton-Valentine leucocidin

Another exotoxin which probably serves to protect *S. aureus* against the defending leucocytes of the host is Panton-Valentine leucocidin (Woodin 1960; Woodin 1970). The leucotoxic action of this non haemolytic leucocidin requires the synergistic action of two proteins, the F and S components (Woodin 1970). When exposed to a mixture of F and S components, susceptible leucocytes exhibit an increased permeability to cations but larger molecules such as phosphates, nucleotides and reducing sugars are retained within the cell (Woodin 1972). This causes efflux of potassium and magnesium ions from the cells and impairs the function of the sodium-potassium pump, which is accompanied by influx of sodium and calcium ions into the leucocytes, with osmotic swelling of cells (Woodin 1965). It has been suggested that leucocidin enhances staphylococcal invasiveness by allowing the organism to resist phagocytosis (Rogers and Tomsett 1952).

Protein A

Protein A is a widely distributed cell wall protein of many strains of *S. aureus* which is covalently bound to the peptidoglycan of the cell wall (Sjoquist *et al* 1972; Sjodahl 1977). Its main feature of interest is its high

affinity for the Fc region of many classes of IgG from a wide range of mammalian species (Goding 1978). Five repeated domains which bind the immunoglobulin molecules protrude from the cell surface (Lofdahl *et al* 1983). These complexes can activate both the classical and alternative pathways of complement (Dossett *et al* 1969). The presence of protein A on the surface of the staphylococcus has also been shown to impair its phagocytosis by human polymorphonuclear leucocytes *in vitro* (Peterson *et al* 1977a). It impairs opsonophagocytosis of bacterial cells and can induce hypersensitivity and histamine release from basophils (Fosgren *et al* 1983). Evidence for a role for protein A in pathogenesis is not definitive, although the level of its expression has been shown to correlate with resistance to opsonophagocytosis *in vitro* (Peterson *et al* 1977a). Protein A deficient mutants were less virulent than wild type strains in a peritonitis and skin abscess model (Patel *et al* 1987). A mutant which expresses high levels of protein A was found to be virulent for mouse mammary gland. Protein A was found to be virulent for mouse mastitis when it is expressed at a sufficiently high level (Foster *et al* 1990).

Disease caused by *S. aureus* may be the result of tissue invasion by a variety of extracellular products. Of these, three toxins are known which have closely related clinical features. Enterotoxins produce food poisoning, exfoliative toxins cause staphylococcal scalded skin syndrome and toxic shock syndrome toxin may cause toxic shock syndrome.

Enterotoxins

The enterotoxins of *S. aureus* form a group of five serologically distinct extracellular proteins, designated A, B, C, D and E (SEA - SEE) (Bergdoll *et al* 1973). SEC is further broken down into three subtypes (SEC_1, SEC_2 and SEC_3) that have minor differences in serological reactions. They are recognised as the causative agents of staphylococcal food poisoning (Dack *et al* 1930). Ingestion of preformed toxin in contaminated food leads to the rapid development (2-6 h) of the symptoms of vomiting and diarrhoea that characterise staphylococcal food poisoning. The toxin is absorbed and reaches the central nervous system via the systemic circulation (Sugiyama and Hayama 1965). It is the action of the toxin on the vomiting centre of the central nervous system that results in often violent gastrointestinal tract manifestations of disease. Besides being emetic toxins, they have potent immunological activities such as stimulation of T-cell proliferation and induction of interleukin-1 and tumour necrosis factor (Parsonet *et al* 1985; Fast *et al* 1989). The enterotoxins are often called neurotoxins because of their effect on the nervous system (Freer and Arbuthnott 1986).

Exfoliative Toxin

It is produced by certain strains of *S. aureus* and is controlled by a plasmid. It causes exfoliation of the skin of infected children, a clinical condition known as "scalded skin syndrome" (Melish *et al* 1972; Piemont

et al 1984). The disease is characterised by a region of erythema which usually begins around the mouth and in 1-2 days, extends over the whole body. The most striking feature of the disease, is that the epidermis although apparently healthy, can be displaced and wrinkled like the skin of a ripe peach by the slightest pressure. Soon large areas of the epidermis become lifted by a layer of serous fluid and peel at the slightest touch (Melish and Glasgow 1970). The mechanism by which exfoliation achieves intra-epidermal splitting is not known, but all available evidence suggests that it is an extracellular, not cytotoxic process (Elias *et al* 1977). Two serotypes of the toxin, ET A and ET B have been recognised (Kondo *et al* 1975); ET A is encoded chromosomally while ET B is plasmid encoded (O'Toole and Foster 1986). Certain strains produce only a single form of ET, while others produce both toxins (Kondo *et al* 1975).

Toxic Shock Syndrome

The syndrome is characterised by fever, rash, hypotension and eventual desquamation of the skin of the palms of the hands and the soles of the feet (Todd *et al* 1978). It was thought that the persons most at risk were young menstruating women who used tampons (Davis *et al* 1980; Shands *et al* 1980). It is now known that toxic shock may occur in persons of any age, race or sex, provided there is an associated staphylococcal infection. There is broad agreement that the multisystem effects observed in toxic shock syndrome patients are induced by toxic shock

syndrome toxin-1(TSST-1)(Bergdoll *et al* 1981; Schlievert *et al* 1981).

The multisystem effects that typify TSS point to an indirect action of TSST-1, followed by the release of mediator substances such as interleukins and tumour necrosis factor (Parsonnet *et al* 1985; de Azavedo *et al* 1988). Occasionally clinical strains lack the ability to produce TSST-1; this gives rise to speculation that toxic factors other than TSST-1 e.g. enterotoxin B (Crass and Bergdoll 1986) may be able to induce physiological and pathological changes similar to TSST-1.

Role of staphylococcal virulence factors in disease

Staphylococcal disease in man encompasses a spectrum of clinical manifestations (eg. toxin-mediated syndromes, contiguous infections, bacteraemia, asymptomatic colonisation) depending on the properties of the strain of *S. aureus*, the site of infection and the degree to which host defences are compromised. The role of *S. aureus* virulence factors in tissue damage and infection has not been studied in much detail. Since several toxins are elaborated in staphylococcal lesions, interactions between the toxins might occur and need to be taken in to account, before the overall contribution to the virulence factors can be determined.

S. aureus isolates from septicaemia showed a higher incidence of enterotoxins A,B and C production (Humphreys *et al* 1989), indicating

that the enterotoxins have a role to play in *S. aureus* infections other than food poisoning. Christensson and Hedstrom (1986) found an inverse relationship between α toxin production and TSST-1 production.

1.3.3 Antibiotic resistance in *S. aureus*

Staphylococci are widely distributed in nature and are among the commonest human parasites. One of the most threatening problems is the great ability of staphylococci to become resistant to most antibacterials introduced to combat the organism. Penicillin was introduced in the early 1940's, and at that time, less than 1% of *S. aureus* strains isolated showed resistance. By the late 1940's penicillin resistance in *S. aureus* had increased, such that by 1946, 60% of hospital strains in the UK were penicillin resistant (Barber and Rozwadowska-Dowzenko 1948). Streptomycin and tetracycline were introduced towards the end of the 1940's. Streptomycin resistance rapidly appeared (Demerec 1948) but tetracycline was often successfully used to treat infections caused by penicillin-resistant staphylococci.

During the 1950's, there was an increase in the incidence of multiple antibiotic resistant strains and virulent penicillinase-producing strains. A new virulent phage type 80/81, which could spread extensively in hospitals and was penicillin resistant was observed in Australia (Rountree and Freeman 1955). Many strains had a broad range of resistances to four or more antibiotics (Bulger and Sherris 1968).

Methicillin and cloxacillin were introduced early in the 1960's, and there was a general decline in the prevalence of multiple resistant *S. aureus* strains during the mid and late 1960's (Bulger and Sherris 1968). Staphylococcal resistance to methicillin and cloxacillin did not emerge immediately, as seen after the introduction of other antibiotics. However, in Europe, methicillin resistance problems were increasingly reported and the strains were also resistant to tetracycline, erythromycin and kanamycin (Benner and Kayser 1968; Rosendal 1971). Britain also experienced an increase in methicillin resistance, reaching 5% in 1969 (Parker and Hewitt 1970). Multiple antibiotic resistant *S. aureus* were still common at the end of the 1960's although they were gradually declining. Cloxacillin could be used to treat infections caused by these organisms and vancomycin and gentamicin were used to treat MRSA infections (Pittman *et al* 1965; Benner and Marthland 1967).

The declining incidence of multiple antibiotic resistance observed in the 1960's continued during the 1970's, not only in Britain (Phillips 1979) but in other countries as well (Plorde and Sherris 1974; Rosendal *et al* 1977). Gentamicin had been used for almost a decade and resistance had been rare. Then in 1976 gentamicin resistant strains emerged (Blint 1976; Speller *et al* 1976).

An upsurge of methicillin - resistant *S. aureus* was noted in the 1980's causing clinical problems since they were resistant to many antibiotics

(Marples *et al* 1986). Some strains of *S. aureus* showed resistance to as many as 20 antimicrobial agents, including antiseptics and heavy metal ions (Lyon and Skurray 1987). Novel MRSA strains emerged which could be distinguished from other MRSA by their antibiotic resistance profile, location of resistance genes and their phage type (Cookson and Phillips 1988, 1990). During the early 1980's a new group of quinolone antibacterials were being developed which were found to be active against *S. aureus* and were useful in the treatment of infections caused by MRSA (Neu 1987; Smith *et al* 1987). This new group of quinolone derivatives were termed the 4-quinolones.

1.4 Quinolone Antibacterials

1.4.1 History

The quinolone antibacterial agents are similar to trimethoprim and the sulphonamides, in the sense that they are chemically synthesised compounds and were not naturally isolated from moulds or fungi. The antibacterial activity of nalidixic acid, the progenitor of the 4-quinolones, was fortuitously discovered during chloroquine synthesis (Lesher *et al* 1962). Nalidixic acid has low activity against Gram negative bacteria along with poor pharmacology plus reports of clinical failures and development of resistance (Ronald *et al* 1966), it soon fell out of favour. Development of more potent and wider spectrum derivatives has continued with the introduction of the new fluoroquinolones such as

ofloxacin, norfloxacin and ciprofloxacin. In contrast to nalidixic acid originally used for urinary tract infections, the newer agents had a broader spectrum of activity. Whereas oxolinic acid, norfloxacin and ciprofloxacin are true quinolones in the chemical sense, other derivatives are not; for example nalidixic acid and enoxacin are strictly naphthyridines. For the sake of simplicity the collective term 4-quinolone was proposed as a generic name for all these bacterial agents, despite the fact that this does not necessarily conform to strict chemical terminology (Smith 1984).

1.4.2 Mode of action

Bacteria are confronted with a major problem, because each contains a chromosome that is composed of double stranded DNA 1300 μm long and yet the average bacterium is only 2 μm long and 1 μm wide. Worcel (1974) studied how the chromosome is packed in *Escherichia coli* and found that it is subdivided into about 65 regions which he termed domains. Each domain, on average about 20 μm long, is attached to an RNA core and the size of each domain is reduced by being wound up tightly through the introduction of supercoiling. Such supercoiling occurs in *E. coli* and most other bacteria against the normal direction of the helical state of DNA in its linear form (Wang 1974) and is termed negative supercoiling. Each domain is supercoiled in an event during which the chromosome is transiently nicked. The enzyme that nicks double-stranded chromosomal DNA, is termed DNA gyrase (Gellert *et al*

1976). DNA gyrase is composed of four subunits, two A and two B (Higgins *et al* 1978) and there seems to be one tetramer per domain. It is believed that the A subunit first introduces a nick into each strand of the double stranded DNA (Gellert *et al* 1977). The nicks are staggered by four base pairs (Morrison and Cozzarelli 1979). The DNA is then thought to be supercoiled by the B subunits which utilise a molecule of ATP for each twist removed. Finally, the resultant negative supercoils are locked into each domain by the A subunits, which are thought to seal the nicks that they first introduced (Gellert *et al* 1977). It was proposed (Crumplin and Smith 1976; Gellert *et al* 1977) that the 4-quinolones prevent the A subunits of DNA gyrase from finally sealing the staggered nicks they first introduced into chromosomal DNA. However, it is known that mutations that affect the B subunit of DNA gyrase change bacterial sensitivity to the 4-quinolone antibacterials (Inoue *et al* 1978; Smith 1984) and so the 4-quinolones would seem to affect not only the A subunits of DNA gyrase but also the B subunits.

1.4.3 Resistance

The 4-quinolones have not yet been affected by plasmid mediated resistance, which is exceptional among antibacterial agents. As a result of this, the frequency of clinical resistance to the 4-quinolones is much less than that seen with other major groups of antibiotics and chemotherapeutic agents (Burman 1977).

At present plasmid-mediated resistance to the modern 4-quinolones has not been identified in clinical isolates. There have been reports of plasmid-mediated nalidixic acid resistance in *Shigella dysenteriae* strains isolated in Kashmir (Panhotra *et al* 1985) and Bangladesh (Munshi *et al* 1987). However, these reports were inconclusive and it was suggested that the plasmid carried mutator genes that increased the frequency of chromosomal mutations to nalidixic acid (Crumplin 1987).

As plasmid-mediated quinolone resistance has yet to be identified in a clinical strain, chromosomal mutations are the only mechanism by which bacteria are able to develop resistance to the 4-quinolones during therapy. Chromosomally mediated resistance to the 4-quinolones can occur by one of two mechanisms - either an alteration in the target enzyme, DNA gyrase, or a mutation that reduces drug accumulation.

In most species investigated so far, high level resistance to all 4-quinolones appears to be conferred by mutations in the *gyr A* gene which codes for the A subunit of DNA gyrase. *Gyr A* mutations confer high level cross resistance to all 4-quinolones, but do not seem to be associated with resistance to other, unrelated antibacterial agents. Such mutations have been identified in *E. coli* (Hooper *et al* 1986; Nakamura *et al* 1989), *Pseudomonas aeruginosa* (Robillard and Scarpa 1988), *Haemophilus influenzae* (Setlow *et al* 1985) and *Serratia marcescens* (Fujimaki *et al* 1989). Mutations in the B subunit of DNA gyrase coded by

the *gyr B* gene have shown to cause 4-quinolone resistance in *Ps. aeruginosa* and *E. coli* (Nakamura *et al* 1989; Yoshida *et al* 1990b). Most of the investigations on the effect of 4-quinolones on DNA gyrase activity have been carried out on Gram negative bacteria with few investigations on Gram positive organisms. It has been suggested that mutations in *gyr A* gene of *S. aureus* are responsible for ciprofloxacin resistance (Sreedharan *et al* 1990).

The second mechanism of 4-quinolone resistance - reduced drug accumulation, causes low level resistance to quinolones and structurally unrelated drugs such as beta-lactam agents, tetracycline and chloramphenicol. Mutations in *E. coli* which confer resistance to the 4-quinolones by an impermeability mechanism are associated with outer membrane porin F (omp F)(Hirai *et al* 1986; Hooper *et al* 1989). An energy-requiring saturable quinolone efflux system at the inner membrane of *E. coli* has been described (Cohen *et al* 1988). The efflux system was identified with the use of everted (inside out) inner membrane vesicles, that accumulated norfloxacin when lactate was added to allow generation of a proton motive force. Since the membranes were everted the observed drug accumulation reflected drug efflux in an intact cell. A mutation in the so-called *nor A* gene has been linked to quinolone resistance in *S. aureus*. The gene seems to code for a protein which is involved in some essential physiological activity in the cell. As a point mutation of the *nor A* gene caused quinolone

resistance by preventing cell accumulation of quinolone antibacterials, it was suggested that the function of the *nor A* gene product is to transport hydrophilic quinolone molecules across the cell membrane (Ohshita *et al* 1990; Yoshida *et al* 1990a).

1.4.4 Relationship between resistance and virulence

Many virulence factors are known to be encoded by plasmids eg. toxins, adhesion factors etc. In a study of enterotoxigenic *E. coli*, 72% were resistant to one or more antibiotics and 44% were resistant to four or more antibiotics (Echeverria *et al* 1987). In transfer experiments, none of the recipients acquired toxigenicity without antibiotic resistance. Genes coding antibiotic resistance and enterotoxins production could be transferred together (Echeverria *et al* 1978). Enteropathogenic *E. coli* adhere to HeLa cells in two different ways - localised adherence (LA) and diffuse adherence (DA). Naturally occurring plasmids encoding for both LA and antibiotic resistance have been recognised in *E. coli* (Laporta *et al* 1986). Curing by acridine orange showed that both features were lost simultaneously. Lacey and Chopra (1975) suggested that acquisition of resistance plasmids by *S. aureus* is associated with a decrease in virulence.

The situation is different in bacteria possessing chromosomal mutations conferring antibiotic resistance. Chromosomal mutations occur at a frequency of 10^{-6} to 10^{-8} per cell as a result of spontaneous mutations in

chromosomal genes encoding the target site, or affecting access to the target site. In practice, such mutations are found less frequently in natural bacterial populations which may be attributed to the fact that mutations reduce the fitness of bacteria to survive, i.e. that they lack pathogenicity (Smith 1983; Saunders 1984).

The effect of chromosomal mutations conferring antibiotic resistance on bacterial virulence has been demonstrated. An example is the alteration in outer membrane porins of Gram negative bacteria which are responsible for movement into bacteria of nutrients, ions and certain antibiotics. A complex control mechanism regulates the quantitative production of porins and affects their sensitivity to various antibiotics such as the quinolones, chloramphenicol, tetracycline and beta-lactams. Mutations which affect the transcriptional regulator of this mechanism, Omp R, resulted in strains with markedly reduced virulence (Dorman *et al* 1989). Cyclic AMP and the cAMP receptor protein are essential for transcription of genes and operons involved in catabolite transport and breakdown. The cAMP levels in cells influence synthesis of fimbriae and flagella and manufacture of some OMPs. Deficiency in cAMP can be associated with β-lactam resistance. Mutations in *Salmonella typhimurium* causing a loss of cAMP production lead to avirulent strains (Curtis and Kelly 1987). In another study, gentamicin resistant strains of *Ps. aeruginosa* showed a significant decrease in virulence. They had slower growth rates, and two of the mutant strains had uncapped

lipopolysaccharide (Bryan *et al* 1985). The possession of fully capped lipopolysaccharide (smooth LPS) is a well known virulence factor in Gram negative bacteria (Stephen and Pietrowski 1981). These examples of chromosomal mutations show that genetic changes which are known to be related to antibiotic resistance (eg alteration in OMPs) are associated with decrease virulence.

Exposure of *S. aureus* to increasing concentrations of gentamicin resulted in bacteria that grew as small nonhaemolytic colonies and did not produce coagulase, deoxyribonuclease and mannitol (Musher *et al* 1977). Penicillin resistant *S. aureus* strains showed reduced pathogenicity in mice and reduced production of coagulase and protein A (Rake *et al* 1944; Blair *et al* 1946). A similar situation was observed in methicillin resistant staphylococci i.e. a reduction in virulence in some of the resistant variants (Barber 1961; Knox and Smith 1961). Some methicillin-resistant *S. aureus* had reduced production of protein A and clumping factor (Lacey *et al* 1986). Epidemic MRSA produced low levels of protein A and high levels of coagulase, whereas sporadic strains produced high levels of protein A and a wide variety of coagulase reactions (Roberts and Gaston 1987). Another study showed that MRSA produced significantly more coagulase than MSSA and that enterotoxin A was produced by MRSA and not by MSSA (Jordens *et al* 1989). MRSA were more likely to be α haemolysin producers than MSSA (Coia *et al* 1992). Rifampicin-resistant mutants of *S. aureus* exhibited altered

production of virulence factors and were less virulent for mice (Ai-Ani *et al* 1991).

4-quinolone resistant *Ps. aeruginosa* were obtained from parental strains by serial transfer through subinhibitory concentrations of quinolones. The resistant mutants exhibited reduced virulence for mice and also had increased sensitivity to aminoglycosides (Ravizzola *et al* 1987). Mutants of *E. coli* which were resistant to 4-quinolones failed to haemagglutinate as a result of a lack of surface pili (Crumplin 1990). It was suggested that these 4-quinolone resistant *E. coli* may be less capable to colonise the urinary tract and initiate an acute infection (Crumplin 1990). In some strains of *S. aureus* resistant to ofloxacin, there was a loss of production of coagulase (Smith 1990). In this study however, only a small number of mutants were tested for coagulase production and they were tested by the slide coagulase test, which is known to detect only bound coagulase production (Smith, personal communication). Gemmell and Felmingham (1990) observed that low level quinolone resistance in *S. aureus* and *Ps. aeruginosa* resulted in a depression of respiratory burst in PMN and that high level quinolone resistance resulted in a potentiation of respiratory burst.

Despite these few examples, such changes in pathogenicity associated with the development of antibiotic resistance has hardly been studied. If 4-quinolone - resistant bacteria are less pathogenic as proposed (Smith

1983), then this might represent an important counterbalance to the development of resistance. Studies which examine the pathogenicity of 4-quinolone-resistant mutants would help to assess both the likelihood of such organisms initiating an infection, or sustaining an established infection if the resistance-conferring mutations occur during the course of therapy (Crumplin 1990).

1.5 Investigating the role of microbial products in pathogenicity

1.5.1 Studying Pathogenicity

The role of a microbial product in the pathogenicity of an organism can be studied when the infecting organism secretes only that product, as is the case with *Clostridium botulinum* (Pappenheimer 1965). However, the situation is more complex when the infecting organism secretes more than one product, as with the staphylococci. In these cases, various approaches have been taken to elucidate the role of each of the microbial products. A direct approach has been to isolate and purify each product and to investigate its effect *in vitro* and *in vivo*. Another method has been to compare virulent and avirulent strains or, strains deficient or enhanced in the production of particular toxins. They are compared both *in vitro*, based on their biochemical characteristics and *in vivo*, based on the infection they produce in laboratory animals (Shibl 1983).

Isolation and purification of determinants

Two methods have been used for identifying virulence determinants, chemical purification and genetic manipulation. The second now takes precedence. The chemical approach was first used for the classical bacterial toxins. Culture filtrates were fractionated employing toxicity as an assay until purity was achieved. Neutralisation of toxicity by specific antibody sealed the proof of causation. Once identified the determinant is investigated *in vivo* and *in vitro* (Roth 1988). Genetic manipulation can be used to identify virulence determinants and pinpoint their active sites. Cell components of pathogens that previously were not readily available e.g. those of *Treponema pallidum,* can be produced in sufficient quantity for a representative demonstration of biological activity and influence on virulence (van Emden *et al* 1983).

Comparison of virulent and avirulent strains

The multifactorial nature of pathogenicity can be analysed more effectively by comparing strains of high and low virulence than by observations on a single strain. The presence or absence of a particular virulence determinant has been a matter of chance. Now, genetics is widely used for producing strains of differing virulence. Classic methods of genetic analysis including spontaneous or induced mutation and the standard techniques of gene transfer have been used successfully to study virulence factors. It is possible to compare two homogenic microorganisms which differ from each other in only a single

determinant of pathogenicity (Patel *et al* 1987; Phonimdaeng *et al* 1990). Strains showing high and low virulence should be compared *in vitro* as well as *in vivo* (Smith 1989).

1.5.2 Effect of antibiotics on expression of virulence determinants

The use of antibiotics that have the ability to inhibit production of virulence factors without interfering with bacterial growth is one approach to the study of the role of microbial products. The activity of antibiotics is usually expressed in terms of concentrations that either inhibit or kill microorganisms *in vitro,* or that prevent or treat experimental infection in laboratory animals i.e. the minimum inhibitory concentration (MIC). There has been interest in the effects of sub inhibitory concentrations of antibiotics on bacterial cells. These include changes in cell morphology, ultrastructure, rate of multiplication and secretion of microbial products (Lorian 1980). However, the MIC specifies the minimum inhibitory concentration which keeps the bacterial inoculum below the threshold of visibility at the time of inspection in the particular environment of the test (Greenwood 1976b). Therefore it varies with the size of the bacterial inoculum, incubation period and the medium used. Examining the behaviour of strains in the presence of sub-MIC levels of antibiotics over short periods of time will not fulfill the conditions used to determine the MIC. This limitation should be noted. Also, when sub-MICs are used in *in vivo* models, inaccuracies may occur and should be considered.

Effects on growth rate

Abraham (1949) recognised three effects of sub-MIC antibiotic concentrations; a prolongation of the lag phase, reduction in the rate of logarithmic phase and diminution in the size of the bacterial population reached in the stationary phase. For a given antibiotic one or all of these effects could be observed. Subsequent reports confirmed that sub-MIC antibiotic concentrations significantly inhibited growth (Greenwood 1976a; Shah *et al* 1976).

Effects on adherence

As previously mentioned, adhesion of microorganisms is an essential event in pathogenicity. Sub-MIC levels of antibiotics may alter the ability of various bacteria to adhere to host cells. Sub-MIC levels of penicillin reduced the ability of resting-phase *Streptococcus pyogenes* to adhere to human oral epithelial cells and yet adherence of dividing *Strep. pyogenes* remained unchanged (Alkan and Beachey 1978). Sub-MIC levels of clindamycin increased the adherence of dividing *Strep. pyogenes*, which is due to an alteration in the conformation of the surface of streptococci, so that previously buried structures become exposed. In *S. aureus* sub-MIC levels of antibiotics effect adherence to buccal epithelial cells. Lincomycin and clindamycin decreased the adherence of this organism, whereas enhancement in adherence was shown in the presence of penicillin (Shibl 1985). Sub-MIC levels of penicillin, streptomycin, gentamicin, chloramphenicol and tetracycline

markedly suppressed the adherence of *Escherichia coli* to human buccal epithelium. This loss in adhesion appears to be a result of the inhibition of the formation of surface adhesins rather than to the loss of adhesion once it has formed (Beachey *et al* 1980). The effect of sub-MICs of antibiotics on the interaction of *S. aureus* with fibronectin has been shown (Proctor *et al* 1983). Sub-MICs of lincomycin, erythromycin and chloramphenicol decrease fibronectin binding to *S. aureus,* whereas beta-lactam antibiotics and vancomycin enhanced their interaction.

Effects on the production of toxins and enzymes

Toxin biosynthesis can be either inhibited or stimulated when the producer organisms are grown in the presence of sub-MIC levels of antibiotics. The production of protease by *Ps. aeruginosa* is inhibited by tetracycline (Shibl and Al-Sowaygh 1980). Streptomycin inhibited the production of haemolysin by *E. coli* (Shibl and Gemmell 1983). A variety of toxins and enzymes produced by *S. aureus* can be inhibited by sub-MIC levels of various antibiotics. Coagulase, α and δ haemolysin and DNase can be inhibited by lincomycin, clindamycin and fusidic acid (Gemmell and Shibl 1976). Lincomycin stimulates the production of enterotoxin both in *E. coli* and *Vibrio cholera* (Levner *et al* 1977). In *S. aureus* penicillinase is stimulated by erythromycin and clindomycin (Michel *et al* 1980). Penicillin and vancomycin stimulated the production of haemolysin by *Strep. pneumoniae* (Lorian *et al* 1973).

1.5.3 Necessity to study pathogenicity *in vivo*

Most of the difficulties in the attempts to identify virulence determinants have stemmed from two facts. First, as previously mentioned, pathogenicity is multifactorial and loss of any single factor can result in either partial or almost complete loss of virulence (Smith 1958). Second, virulence is only really detectable *in vivo* and is markedly influenced by changes in growth conditions because of the selection of phenotypes (Meynell 1961). The genetic information which determines virulence may be expressed only under conditions of the test for virulence, namely during growth *in vivo.* When microbes are removed from infected animals and grown *in vitro,* the change of environment will induce phenotypes different from those found *in vivo;* i.e. they may lack some virulence determinants. Also, suspected determinants of pathogenicity, indicated by experiments employing *in vitro* grown organisms, may not be produced *in vivo* (Smith 1964).

Staphylococcus aureus grown *in vivo* differ in their overall amino acid composition from cells grown *in vitro* (Watson and Prideaux 1979). There is evidence that fresh clinical isolates of *S. aureus* (Karakawa and Kane 1975) and *S. aureus* grown under *in vivo* cultural conditions (Watson and Prideaux 1979) possess a different antigenic composition compared with the same strain propagated in the laboratory. Freshly isolated human strains of *S. aureus* were more resistant to phagocytosis by polymorphonuclear leucocytes, a property attributed to the production

by the organism of an acidic polysaccharide surface antigen under *in vivo* cultural conditions (Karakawa and Kane 1975).

Animal models

The importance of the use of animal models in the understanding of the pathogenesis of disease states is well established (Dalhoff 1985). Animal studies can be divided into four general categories : basic screening models, *ex vivo* models, monoparametric models and discriminative models (Zak 1980).

Basic screening models include systemic infection, thigh-lesion test, meningitis or pneumonia in mice. Infection is usually induced with an inoculum that ensures death (or thigh swelling) in all the animals. They are most frequently used in the early evaluation of new antibiotics (Zak *et al* 1985). This model only gives a rough estimate of whether an antibiotic is likely to be effective *in vivo*, since there are several inherent limitations e.g. infections are initiated with a large inoculum and are sensitive to the size of the infective inoculum (Nishino and Zak 1985).

Ex vivo models use a foreign body usually implanted subcutaneously, which is subsequently infected with bacteria. These chambers can be constructed so that only a limited number of parameters such as antibiotic selection, nutrient supply and host immune response can be evaluated during a given investigation (Zak and O'Reilly 1991). Their

major drawback is that they represent a relatively artificial setting of infection (Peterson 1986).

Monoparametric models do not estimate survival as in screening models, but rely on different end points such as serial bacterial concentrations in blood or tissues or alterations in bacterial morphology during the course of therapy (Carbon 1990).

Discriminative models attempt to simulate human infections in order to provide insight into both the pathogenesis and treatment of clinically relevant infections. Many criteria for an ideal model have been described (Harter and Petersdorf 1960). The model should be simple and reproducible and should closely reflect the human disease e.g. meningitis, endocarditis, abscesses (intradominal, subcutaneous).

Aims of the thesis

This thesis is going to study the postulate that ciprofloxacin-resistant mutants of *S. aureus* lack pathogenicity. Ciprofloxacin sensitive and resistant strains will be compared to establish any differences in their respective production of virulence factors both *in vitro* and *in vivo*. Since S. *aureus* produces a large number of virulence factors, it was not possible to study all of them in the time available. A small number were chosen to be studied in detail. The ciprofloxacin-resistant strains were obtained from clinical sources but the type of staphylococcal infection

from which the strains were isolated was not known. Therefore, it did not seem appropriate to investigate the production of TSST-1, epidermolytic toxin or the enterotoxins, since they are mainly specific to disease producing strains. Four factors were chosen which are produced by the majority of *S. aureus* strains; coagulase, protein A, α and δ haemolysins. The production of the factors will be monitored in the absence and presence of sub-MIC levels of certain antibiotics. Then, in order to correlate the *in vitro* results with pathogenicity, the ciprofloxacin-resistant mutants will be investigated *in vivo*. The *in vivo* model was chosen for its reproducibility and ease. A model was needed which could indicate the differences in the pathogenicity of the strains investigated. Lethal dose 50 (LD_{50}) calculations were the archetypal measure of bacterial virulence but they are no longer considered ethically acceptable. A subcutaneous abscess model in mice was considered a good alternative and would extend the study of the pathogenicity of the strains beyond the four factors mentioned in the *in vitro* section of the project.

2 Materials and Methods

2.1 Bacterial Strains

Bacterial strains used during the project are listed in table 2. Paired isolates of ciprofloxacin-sensitive and -resistant strains were obtained from the PHLS, Colindale and Royal Infirmary of Edinburgh i.e. where resistance to ciprofloxacin developed during therapy. However, since only two such pairs were acquired as a result of difficulty in obtaining the pre-therapy ciprofloxacin sensitive isolates, mainly clinical ciprofloxacin -resistant strains were used. The ciprofloxacin-resistant strains (MIC ciprofloxacin > 4 mg/L) were obtained from the PHLS, Colindale and Royal Infirmary of Edinburgh. Ciprofloxacin-sensitive laboratory strains and high virulence factor producing strains were acquired as mentioned in Table 2. Ciprofloxacin-resistant mutants of the high virulence factor producing strains were made as described in section 2.4.5.

All isolates were stored at -70°C in broth containing 10% glycerol and were subcultured on to blood agar and incubated overnight immediately before use.

Bacteriophage typing was kindly performed at the Central Public Health Laboratory, Colindale, London.

Table 2. Bacterial Strains

Strain	Characteristics	Origin
E 3T	Standard sensitive	J T Smith, London
Oxford	Standard sensitive	R Paton, Edinburgh
12009	Coagulase producer	C Gemmell, Glasgow
Cowan 1	Protein A producer	C Gemmell, Glasgow
Wood 46	Alpha haemolysin producer	C Gemmell, Glasgow
E5662	Delta haemolysin producer	C Gemmell, Glasgow
cip 63	Ciprofloxacin resistant strain	R Paton, Edinburgh
cip 86	Ciprofloxacin resistant strain	R Paton, Edinburgh
cip 92	Ciprofloxacin resistant strain	R Paton, Edinburgh
cip 103	Ciprofloxacin resistant strain	R Paton, Edinburgh
cip 132	Ciprofloxacin resistant strain	R Paton, Edinburgh
591-89	Ciprofloxacin resistant strain	PHLS, Colindale, London
4953-88	Ciprofloxacin resistant strain	PHLS, Colindale, London
411-87	Ciprofloxacin resistant strain	PHLS, Colindale, London
8984-88	Ciprofloxacin resistant strain	PHLS, Colindale, London
5538-88	Ciprofloxacin resistant strain	PHLS, Colindale, London
417-87	Ciprofloxacin resistant strain	PHLS, Colindale, London
2219	Ciprofloxacin sensitive strain	PHLS, Colindale, London
2221	Cipro resistant mutant of 2219	PHLS, Colindale, London
2222	Cipro resistant mutant of 2219	PHLS, Colindale, London
6989	Ciprofloxacin sensitive strain	Edinburgh (RIE)
3225	Cipro resistant mutant of 6989	Edinburgh (RIE)
12009-3	Cipro resistant 12009	This study
Cowan-3	Cipro resistant Cowan 1	This study
Wood 46-3	Cipro resistant Wood 46	This study
E5662-3	Cipro resistant E5662	This study

All the strains listed are *Staphylococcus aureus* except strains E5662 and E5662-3 which are *Staphylococcus epidermidis*

2.2 Media

2.2.1 Complex Media

The complex media used were Nutrient Broth No.2 (CM67), Diagnostic Sensitivity Test Agar (CM261), Columbia Agar base (CM331B), Brain Heart Infusion Broth (CM225), Tryptone Soya Broth (CM129) (Oxoid, Basingstoke, Hants).

2.2.2 Minimal Media

Single strength minimal salts medium (DM) was prepared as described by Davis and Mingioli (1950). For the preparation of diluents, single strength DM base was distributed in 9.9 ml and 4.5 ml aliquots which were autoclaved at 121°C for 15 minutes.

2.2.3 Preparation of plates

All laboratory media were made up according to the manufacturer's instructions and were autoclaved at 121°C for 15 minutes. The plates were poured while the agar was still molten, each containing approximately 20 ml. With antimicrobial drug sensitivity plates, the agar was allowed to cool to 50°C before the appropriate antimicrobial drugs were added and the plates poured. After setting, the plates were dried, inverted at 50° C for 20-30 minutes.

2.3 Materials

2.3.1 Antibacterial Agents

The antibacterial agents used are listed in table 3. All were supplied sterile and prepared aseptically as listed.

Table 3. Antibacterial Agents

Antibacterial agent	Supplier	Method used to dissolve
Ciprofloxacin	Bayer	NaOH
Enoxacin	Warner Lambert/Parke Davies	NaOH
Methicillin	SmithKline Beechams	Water
Gentamicin	Sigma	Water
Tetracycline	Lederle	Water
Chloramphenicol	Boehringer Mannheim	Alcohol

2.3.2 Buffers

Sodium phosphate buffers and sodium carbonate/bicarbonate buffers were made according to the Data for Biochemical Research (Oxford University Press).

2.4 Methods

2.4.1 Viable Counts

Sterile dilutions were made with 1 in 10 and 1 in 100 dilution steps in single strength DM i.e. 0.5 ml of culture and 4.5 ml diluent or 0.1 ml culture and 9.9 ml of diluent respectively. Suspensions were mixed on a Rotamixer (Hook and Tucker Ltd) and 0.1 ml amounts spread with a

sterile glass spreader onto nutrient agar plates. The plates were incubated, inverted at 37° C for 18 hours, after which time the plates from dilutions yeilding 10-100 colonies were selected for counting. The number of colonies on the plate were counted and the viable count was calculated using the following equation:

$$\text{colony forming units (cfu)/ml} = N \times 10^{a} \times 0.1,$$

where N is the number of colonies on the plate from 10^{-a} dilution and 10^{a} is the dilution factor. All dilutions were plated out in duplicate and a average of the counts from the two plates was calculated.

2.4.2 Minimum Inhibitory Concentration (MIC) Determinations on Solid Media

Oxoid No.2 Nutrient broth (4.5 ml amounts) was seeded with an inoculum from a fresh nutrient agar plate and grown overnight at 37°C. A 1 in 10^4 dilution was prepared by serial dilutions in DM base and 2 µl of this suspension was spotted onto media containing varying concentrations of the drug with a multipoint inoculator (Denley). Plates were incubated for 18 hours. Concentrations were increased by a factor of 2 and the MIC was taken as the first concentration permitting no visible growth. A control plate lacking any drug was used in each case.

2.4.3 MIC Determination in Broth

Dilutions of each drug were made in 1 ml amounts of broth prior to inoculation with 1×10^5 colony forming units (cfu)/strain and incubated

for 18 hours at 37°C to determine their MIC. Concentrations were increased by a factor of 2 and the MIC was defined as the lowest concentration that inhibited visible growth.

2.4.4 Protein Estimations

Protein estimations were performed by the method of Waddell (1956). All samples to be tested were suitably diluted (1:100 or 1:1000) in 50 mM sodium phosphate buffer pH 7.4 and the absorbance measured at 215 and 225 nm. The protein concentration could then be calculated with reference to a standard curve which had previously been prepared. This method has been shown (Hesslewood 1973) to be as precise and more sensitive than the protein estimation of Lowry *et al* (1951).

2.4.5 Mutational resistance to ciprofloxacin

Ciprofloxacin resistant mutants were selected by culturing the strains on increasing concentrations of ciprofloxacin. The bacteria were grown overnight in nutrient broth and then they were centrifuged at 4,500 rpm for 15 minutes. The cell pellet was resuspended in fresh nutrient broth, before being serially diluted to produce 10^{-2} and 10^{-4} dilutions. One hundred microlitre amounts of the neat culture and the two dilutions were spread with a sterile glass spreader on nutrient agar containing ciprofloxacin at 1 , 2 or 4 times the MIC against sensitive parental strains. The plates were incubated overnight at 37°C. Any colonies which grew on the ciprofloxacin containing plates were subcultured onto nutrient

agar plates containing the same amount of ciprofloxacin. The process was repeated, except that the ciprofloxacin resistant mutant was grown overnight and then plated out on higher concentrations of ciprofloxacin.

2.4.6 Growth Rate Determinations

Growth of strains was monitored over a defined period. Overnight cultures of organisms were subcultured in 50 mls of fresh prewarmed broth and incubated at 37°C with shaking. At hourly intervals, samples were removed for viable counts to be taken. In some cases, the optical density (590 nm) was monitored as well. In experiments where the production of toxins or enzymes was being related to bacterial growth, samples were taken at hourly intervals, the optical density at 590 nm read and then the sample was prepared for the assay of the toxin or enzyme in question.

2.4.7 Anaerobic growth of strains

Overnight cultures of organisms were subcultured into nutrient broth, which had been maintained overnight in an anaerobic cabinet. Strains were then incubated anaerobically overnight. The presence or absence of growth was investigated the next day by viable counts as described in section 2.4.1.

2.4.8 Purification of Staphylocoagulase

Staphylocoagulase was purified by a modification of the method of

Igarashi *et al* (1979), by affinity chromatography on bovine-prothrombin Sepharose 4B. CNBr-activated Sepharose 4B (Sigma) was coupled with bovine prothrombin (Sigma) in accordance with the manufacturer's instructions, before being poured into an Econo-Column (Bio-Rad)(0.375 cm^2 x 20 cm). The filtered sterile culture supernatant of strain 12009 - a high coagulase producer, was applied to the column. The column was washed with 50 mM sodium phosphate buffer pH 7.4 containing 1M NaCl and 0.0001% thiomersal (Sigma) (equilibration buffer) until the absorbance at 280 nm returned to zero. The absorbed staphylocoagulase was eluted with 1M NaSCN and 2 ml fractions were collected. These fractions were dialysed overnight against equilibration buffer. The dialysed samples were assayed for coagulase activity employing the standard tube test (Sperber and Tatini 1975). Two hundred microlitre amounts of sample were mixed with 0.2 ml of 1:10 diluted rabbit plasma (Oxoid). The mixture was incubated for one hour at 37°C and examined for the formation of a plasma clot. Then the purified coagulase was concentrated in Amicon Centriprep 10 and Centricon 10 concentrators. Concentrators were used in accordance with manufacturer's instructions. The concentrated sample was stored at -20°C until use.

2.4.9 Quantitative assay for Staphylocoagulase

Staphylocoagulase activity was measured by a modification of the chromogenic substrate assay of Engels *et al* (1981). In the assay, staphylocoagulase activates prothrombin (Blood Products Laboratory,

Elstree London) to staphylothrombin. The thrombin-like activity of this complex cleaves a synthetic tripeptide, Chromozym T H (Boehringer Mannheim) liberating a yellow product, p-nitroaniline and the optical density is measured at 405 nm. The assay was modified and improved to distinguish bound, soluble and combined coagulase production and the modifications will be discussed in detail in section 3.

2.4.10 Measurement of Protein A

Cell bound and extracellular protein A were detected by a modification of the capture ELISA (enzyme linked immunosorbent assay) described by Warnes *et al* (1986). Extracellular protein A was measured from the supernatant of a broth culture and the pellet was processed to yield cell-free lysates. Cell-free lysates were prepared as described by Warnes *et al* (1986), except that after lysostaphin (Sigma) treatment, the lysates were sonicated twice for 30 seconds at amplitude 10 on an MSE Soniprep, while kept on ice. The lysates were centrifuged at 11 K rpm for 45 minutes before the pellets were discarded and the supernatants used as the cell free lysates. The capture ELISA utilised human IgG (Sigma) bound to the plates (Nunc Immuno module polysorp F8, Gibco Life Technologies Ltd). Protein A was detected by incubation with rabbit anti-protein A serum (Sigma) and then with goat (anti-rabbit IgG) IgG-alkaline phosphatase conjugate (Sigma). The bound conjugate was incubated with p-nitrophenylphosphate (Sigma) for 30 minutes at room temperature, and the optical density determined at 405 nm in a Titertek

Multiskan plate reader (Flow Laboratories Ltd). All isolates were tested in duplicate on at least two separate occasions.

The amount of protein A produced by the isolates was related to total cell protein. The protein concentration of the cell-free lysates was estimated by the method of Waddell (1956) described in section 2.4.4. This gave an estimate of the total cell protein. Each protein A ELISA contained standard protein A (Sigma) concentrations, so that the amount of protein A in the samples could be calculated from a standard curve. Then the protein A in the samples was calculated in relation to total cell protein. Extracellular protein A was measured in the supernatant of the culture and cell bound protein A was measured from the processed cell pellet i.e. cell free lysates.

2.4.11 IgG Column for elimination of Protein A

α and δ haemolysins were measured by two separate ELISAs. Unfortunately, major interferences occurred as a result of IgG-binding protein A which is also present in the culture supernatant to be tested. This problem was resolved by passing the supernatants through a CL-4B Sepharose-IgG column, which adsorbed any protein A present (Berdal *et al* 1981). The adsorption column was prepared as described by Berdal *et al* (1981) and also according to the manufacturer's instructions. Filtered supernatants were prepared as described by Berdal *et al* (1981), except that the strains were grown in Tryptone Soya broth supplemented with 0.3%

yeast extract which is reported to enhance haemolysin production (Goode and Baldwin 1973). The supernatants were then passed through the adsorption column. Once supernatants had been passed through the column, they were tested for the presence of protein A by the protein A ELISA described in section 2.4.10. All the samples should have been free of protein A and any that were not, were passed through the column a second time. The column was eluted after 10 samples had been passed through it in order to remove all the bound protein A. This was achieved by flushing through 0.1M Glycine and then 0.05M Borate buffer for 30 minutes (Berdal *et al* 1981).

2.4.12 Measurement of α haemolysin

α haemolysin was detected and quantified by an indirect ELISA. Microtitre plates (Nunc Immuno module polysorp F8, Gibco BRL Life Technologies) were filled with 100 µl of either sample or pure α haemolysin (Gibco) serially diluted in coating buffer (50 mM sodium carbonate/sodium bicarbonate buffer pH 9.6 and 0.02% sodium azide). The plates were left overnight at room temperature. The wells were decanted and washed four times with phosphate buffered saline containing 0.1% Tween 20 (PBS-T). They were then filled with 100 µl of 1:100 dilution of α haemolysin antisera (gift from Mr D Pickard, Wellcome Research Laboratories, Beckenham, Kent) and incubated at room temperature for 90 minutes. The wells were decanted and washed as before, and then filled with 100 µl of 1:4000 dilution of goat (anti-rabbit

IgG) IgG-alkaline phosphatase conjugate (Sigma) and incubated for 90 minutes at room temperature. The wells were decanted and washed, and then filled with 1 mg/ml p-nitrophenyl phosphate (Sigma) which was dissolved in substrate buffer (50mM carbonate buffer pH 9.6) and incubated for 120 minutes at room temperature. The optical density at 405 nm was read on a Titertek Multiskan plate reader (Flow Laboratories Ltd). All isolates were tested in duplicate on at least two separate occasions.

α haemolysin production was related to viable cells and to total cell protein. Each ELISA plate had standard α haemolysin concentrations, so that the amount of α haemolysin in the samples could be calculated. Viable counts were performed on the cultures by serial dilution with 1 in 10 and 1 in 100 dilution steps in DM. 0.1 ml amounts were spread on nutrient agar plates with a sterile glass spreader. Plates were incubated, inverted at 37°C for 18 hours. Total cell protein concentration was calculated as described in section 2.4.10. Once this, and the viable counts had been calculated, the α haemolysin concentration in the samples could be related to them.

2.4.13 Purification of δ haemolysin

δ haemolysin was purified by the method of Heatley (1971, 1976) from *S. epidermidis* strain E5662 - a strain known to produce large amounts of δ haemolysin. The final precipitate was freeze dried before being stored at

-20° C until use. Haemolytic activity of the purified toxin was measured by the method of Heatley (1971). Human blood was obtained from the Blood Transfusion Service, Edinburgh. One haemolytic unit was taken as the amount of toxin yielding 50% haemolysis in the standard assay.

2.4.14 Measurement of δ haemolysin

The measurement of δ haemolysin was based on a method described by Scheifele *et al* (1987) employing an indirect ELISA. Samples and purified δ haemolysin were serially diluted in coating buffer (50 mM sodium carbonate/sodium bicarbonate buffer pH 9.6 and 0.02% sodium azide) and 100 µl of the samples were added to the wells of a microtitre plate (Nunc Immuno Module polysorp F8, Gibco BRL Life Technologies). Plates were incubated overnight at room temperature. Plates were washed with phosphate buffered saline containing 0.1% Tween 20 (PBS-T) four times. δ haemolysin antiserum (gift from Mr D Pickard, Wellcome Research Laboratories, Beckenham, Kent) was added (1:200 dilution) and incubated for 90 minutes at room temperature. The wells were washed as before and then filled with 100 µl of 1:4000 dilution of goat (anti-rabbit IgG) IgG-alkaline phosphatase conjugate (Sigma) and incubated for 90 minutes at room temperature. Finally the substrate, p-nitrophenylphosphate conjugate (Sigma) dissolved in coating buffer, was added to the wells and incubated for 120 minutes at room temperature. Optical density (405 nm) was read by a Titertek Multiskan plate reader (Flow Laboratories Ltd). All isolates were tested in duplicate

on at least two separate occasions. δ haemolysin production was related to viable cells and to total cell protein as described in section 2.4.12 for α haemolysin production.

2.4.15 *In vivo* pathogenicity of the strains

The *in vivo* pathogenicity of the strains was established employing a subcutaneous abscess model in mice (Bunce *et al* 1992). The bacterial culture was grown to an optical density (590 nm) of 0.2 which is equivalent to 10^8 cfu/ml, as determined from a standard curve which had been prepared previously. Five hundred microlitres of this was mixed with 190 µl Cytodex-1 microcarriers (Sigma), which had been prepared according to the manufacturer's instructions. Brain Heart Infusion broth (310 µl) was added to the inoculum mixture before it was mixed thoroughly. Two hundred microlitre amounts of this mixture were drawn up into a 1 ml syringe (Beckton/Dickinson) with a 25 gauge needle. A separate syringe was prepared for each mouse. The inoculum was injected subcutaneously into C3H mice (male, 8 weeks old). The fur had been shaved off the rear flank where the bacterial inoculum was to be injected, to enable easy viewing of the abscesses. The bevel of the needle faced upwards when the needle was inserted, to ensure spherical abscess formation and also consistency of injections. The initial bacterial inoculum was measured to ensure that 1x 10^7 cfu/ml was injected. This was achieved by viable counts as described in section 2.4.1. Each mouse was identified by a notch on the ear, with each of the 4 mice in a cage

having a different marking. The syringes were coded so that the injections were randomised and 'blind'. The development of the abscesses was monitored over a period of 10 days, with the size and severity of the abscesses being noted. The code was not consulted until the end of the experiment to ensure unbiased monitoring of the abscesses. Ten mice were used for each test strain. The volumes of the abscesses were calculated using the equation for a spherical ellipsoid ($\pi/6 \times l \times w^2$), taking l as the longest measurement and w as the shortest.

The method was reproducible, in the sense that abscesses produced by a particular strain in each of 10 mice, were of similar size and the standard error for the mean of the daily volumes was small.

2.4.16 Estimation of viable bacteria in abscesses

Abscesses were removed from dead mice so that an estimate of viable bacteria could be calculated. The area surrounding the abscess was wet with alcohol. The skin on three sides of the abscess area was cut and peeled back. The abscess was then cut out and placed in PBS. The abscess was broken up and the mixture was serially diluted with 1 in 10 and 1 in 100 dilution steps in DM. Suspensions were mixed and then 0.1 ml amounts were spread with a sterile glass spreader onto nutrient agar plates. Plates were incubated, inverted at 37°C for 18 hours.

2.4.17 Estimation of ciprofloxacin levels in serum and abscesses of mice

The level of ciprofloxacin in the serum and abscesses of mice was estimated as follows. Known amounts of ciprofloxacin (Bayer UK) were injected subcutaneously and intravenously into mice. Thirty minutes later blood samples were taken ocularly and the serum separated from the blood cells and stored in ice. Three hours later the mice were sacrificed and the abscesses were excised. The blood samples and the abscesses were analysed by HPLC for levels of ciprofloxacin (Les White, Southmead Hospital). The amount of ciprofloxacin injected subcutaneously or intravenously could be related to levels in the blood or in the abscess.

2.4.18 Treatment of mice with ciprofloxacin

Mice were given subcutaneous injections of ciprofloxacin diluted in PBS every two hours on the opposite side from the injection of the bacterial inoculum.

3 Results - Pathogenicity and Ciprofloxacin Resistance

The first section of the study investigated the postulate that ciprofloxacin resistant mutants of *S. aureus* lack pathogenicity. Ciprofloxacin sensitive and resistant strains were compared for their production of virulence factors both *in vitro* and *in vivo*. The production of four virulence factors was studied in detail - the four factors being coagulase, protein A, α haemolysin and δ haemolysin.

3.1 Bacterial Strains

The strains used during the study were selected mainly because of their resistance to ciprofloxacin. Since only two sets of paired isolates of ciprofloxacin sensitive and resistant strains could be obtained, most of the ciprofloxacin resistant strains were compared to sensitive laboratory strains. Four strains were acquired which were known to produce high levels of the four virulence factors which were going to be studied in detail. These strains were used to establish the assay procedures and conditions. The minimum inhibitory concentrations (MICs) of the strains were determined for several antibiotics on both solid media and in broth. A narrow spectrum of concentrations was tested since accurate MICs were needed later in the project (Table 4).

Table 4. Minimum Inhibitory Concentrations of six antibiotics for the Bacterial strains used

	Minimum Inhibitory concentrations (mg/L)					
Strain	Cip	Enox	Meth	Gent	Chlor	Tetra
E3T	0.187	0.75	1	0.187	3	0.5
Oxford	0.187	0.5	1	0.25	3	0.125
12009	0.75	1.5	1	0.187	2	0.187
Cowan 1	0.187	0.75	1	0.187	1.5	0.187
Wood 46	0.187	0.75	1	0.375	2	0.187
E5662	0.187	0.75	3	0.375	1.5	0.187
cip 63	16	48	2	0.5	2	96
cip 86	64	64	1.5	0.5	6	0.5
cip 92	4	6	1	0.5	3	0.375
cip 103	48	48	1.5	0.5	4	0.375
cip 132	32	48	2	0.5	3	0.375
411-87	48	48	2	0.5	3	0.375
417-87	12	24	16	48	4	64
591-89	24	48	12	96	3	8
4953-88	24	32	24	96	24	64
5538-88	24	48	48	0.5	4	96
8984-88	128	128	1.5	0.5	4	0.375
2219	0.375	1	96	0.5	4	128
2221	6	8	96	0.5	4	128
2222	24	32	96	0.5	4	128
6989	0.375	1.5	1	0.375	4	0.5
3225	3	24	1	0.375	4	0.5
12009-3	32	64	1	0.187	2	0.187
Cowan-3	32	64	1	0.187	1.5	0.187
Wood46-3	32	64	1	0.375	2	0.187
E5662 -3	32	64	3	0.375	1.5	0.187

Cip - ciprofloxacin, Enox - enoxacin, Meth - methicillin, Gent - gentamicin, Chlor - chloramphenicol, Tetra - tetracycline

The ciprofloxacin resistant strains were bacteriophage typed, since this is a normal way of distinguishing staphylococcal strains (Table 5). Strains 2219, 2221, 2222 and 5538-88 were indistinguishable MRSA, strains 591-89 and 417-88 were different MRSA. Strains 6989 and 3225, which were a pair of ciprofloxacin sensitive and resistant isolates, were indistinguishable by phage typing suggesting that they were the same strain which had developed resistance to ciprofloxacin during therapy. Strains 2219, 2221 and 2222 had been isolated from a patient over a period of 10 days during ciprofloxacin therapy, were also indistinguishable by phage typing which suggests that they were the same strain which had developed resistance.

3.2 Growth Rate Determinations

Growth rate is important in pathogenicity. A fast growing pathogen can overwhelm the initial, non specific defences and cause disease before the more powerful immune defences can operate fully (Smith 1990). Avirulence can arise from inability of bacteria to grow and divide (Smith 1968). For this reason, the growth rates of some ciprofloxacin sensitive and resistant strains of *S. aureus* were compared.

3.2.1 Growth rates of ciprofloxacin sensitive and resistant strains

The growth rates of some ciprofloxacin resistant clinical isolates were compared with those of ciprofloxacin sensitive laboratory strains, since at the beginning of the project, paired strains of ciprofloxacin resistant and

Table 5. Bacteriophage Typing

Strain	Phage typing pattern
E3T	77 (42E, 84)
cip 63	29, 52, 52A, 42E
cip 86	(53, 83A, 84, 85)
cip 92	NT (95)
cip 103	NT
cip 132	(53, 83A)
411-87	29, 52, 52A, 80, 95
417-87	54, 77 (85)
519-89	85 (83A,)
4953-88	85
5538-88	84 (85)
8984-88	NT
2219	84 (85)
2221	84 (85)
2222	84 (85)
6989	3A
3225	3A

Figures in parentheses Routine Testing Dose (RTD) x 100

sensitive strains were unavailable. Overnight nutrient broth cultures were subcultured into fresh prewarmed broth and the viable count monitored every hour for six hours.

Figures 1 and 2 show the growth curves of the strains grown statically and with aeration. No detectable differences could be seen between the growth rates of the ciprofloxacin sensitive and resistant strains in logarithmic phase.

3.2.2 Growth rates of paired ciprofloxacin sensitive and resistant strains

Some strain series were obtained - a triplet 2219, 2221, 2222 and a pair 6989, 3225. The growth rates of these strains were compared as described above. Figures 3 - 6 show the growth curves of these strains. Again, no detectable differences could be seen between the strains.

3.2.3 Growth rates in mixed cultures

No detectable differences in the growth of the strains could be seen in logarithmic phase but differences might occur in stationary phase, especially if a ciprofloxacin resistant strain is grown in the presence of a ciprofloxacin sensitive strain. Would the resistant strain persist in a mixed culture in the absence of ciprofloxacin? Overnight cultures of a sensitive and a resistant strain were subcultured into fresh nutrient broth. Approximately equal titres of each strain were added to make the mixed culture. The cultures were incubated statically and growth

Figure 1. Growth curves of ciprofloxacin sensitive and resistant strains grown statically

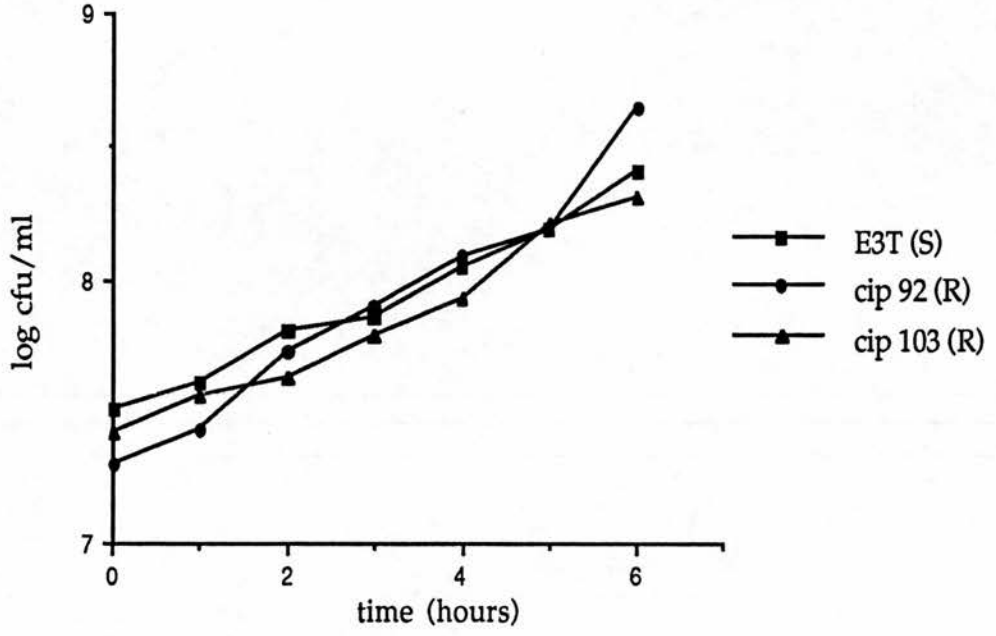


Figure 2. Growth curves of ciprofloxacin sensitive and resistant strains grown with aeration

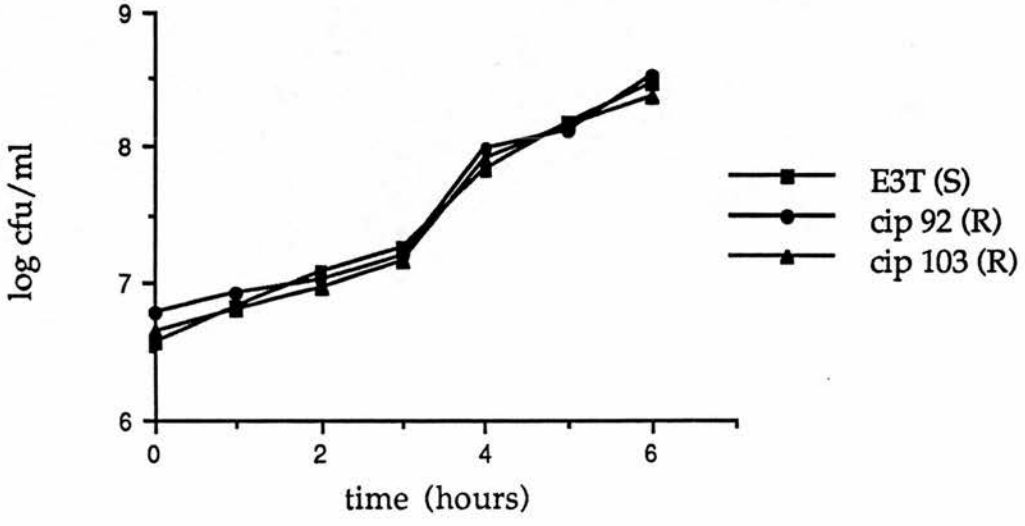


Figure 3. Growth curves of paired ciprofloxacin sensitive and resistant strains grown statically

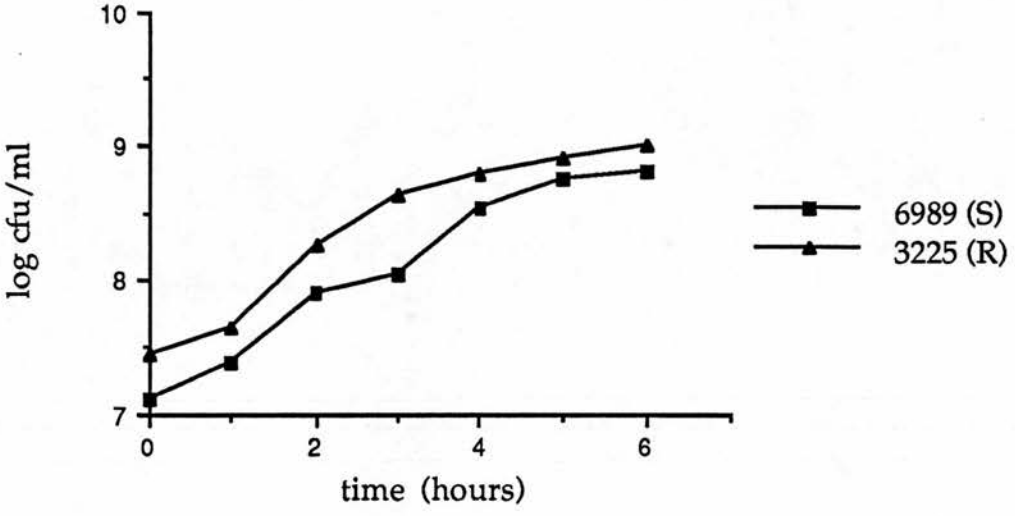


Figure 4. Growth curves of paired ciprofloxacin sensitive and resistant strains grown with aeration

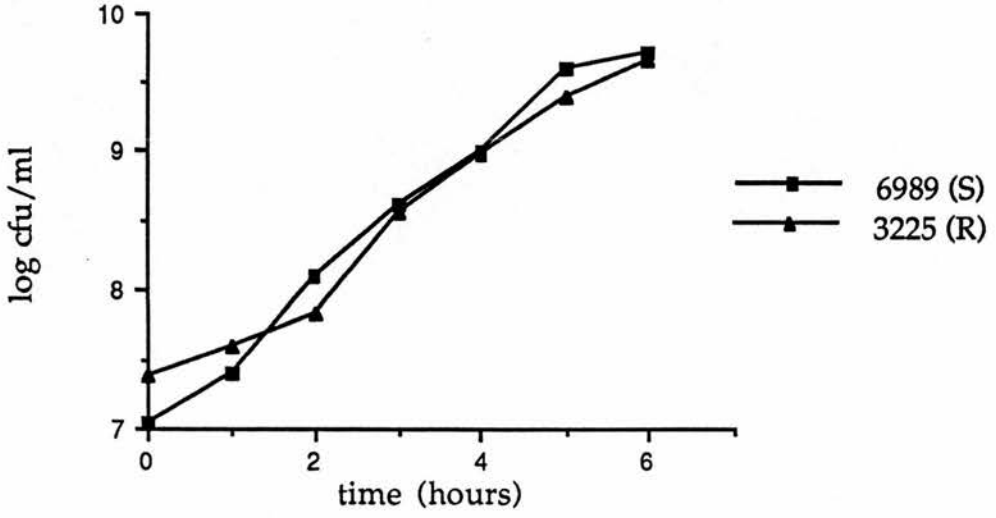


Figure 5. Growth curves of triplet of ciprofloxacin sensitive and resistant strains grown statically

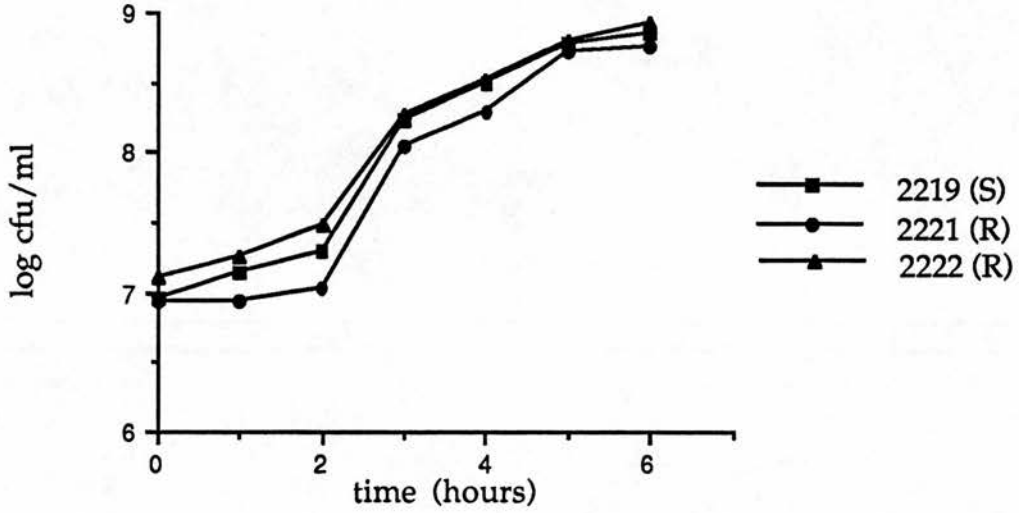
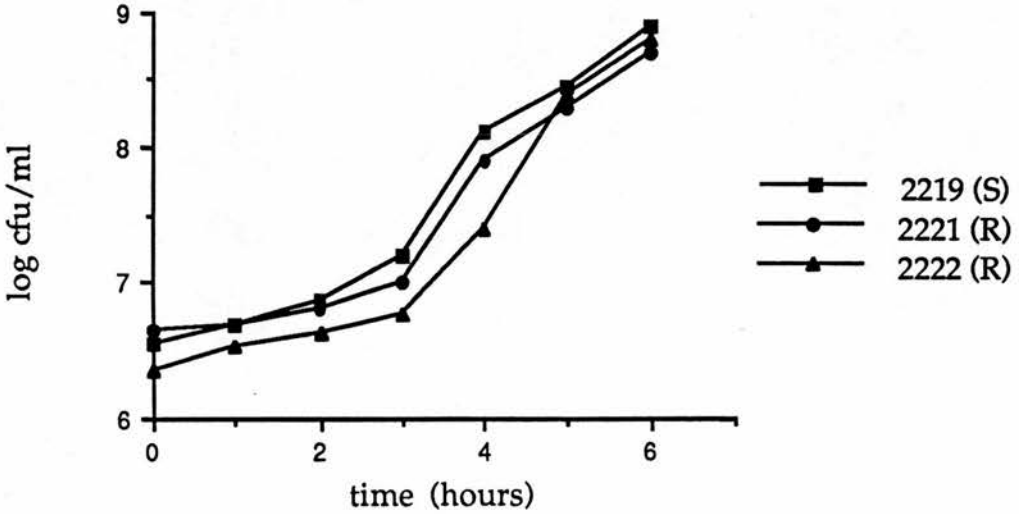


Figure 6. Growth curves of triplet of ciprofloxacin sensitive and resistant strains grown with aeration



monitored over a period of 30 hours by viable counts at regular intervals on nutrient agar containing or lacking 4 mg/L ciprofloxacin. The ciprofloxacin containing plates detected the number of ciprofloxacin resistant bacteria in the mixed culture. In all cases, the ciprofloxacin resistant strains persisted in the mixture; the level of persistence remaining steady over the 30 hours (Figures 7 - 10).

3.2.4 Anaerobic growth of ciprofloxacin sensitive and resistant S. aureus

The ability of bacteria to grow aerobically and anaerobically considerably improves their survival potential and flexibility. It has been shown that alteration in DNA gyrase can cause facultative anaerobes to become strict aerobes (Yoshizawa and Yamamato 1989). Therefore some ciprofloxacin sensitive and resistant strains were grown in an anaerobic cabinet to see if they could still grow anaerobically. Overnight cultures were subcultured into nutrient broth which had been pre-incubated in an anaerobic cabinet overnight. These cultures were incubated anaerobically overnight after which time viable counts were performed. All strains retained the ability to grow anaerobically (Table 6). The strains had also been grown aerobically for comparison.

Figure 7. Growth curves of a ciprofloxacin sensitive strain (E3T), a ciprofloxacin resistant strain (cip 103) and a mixture of the two

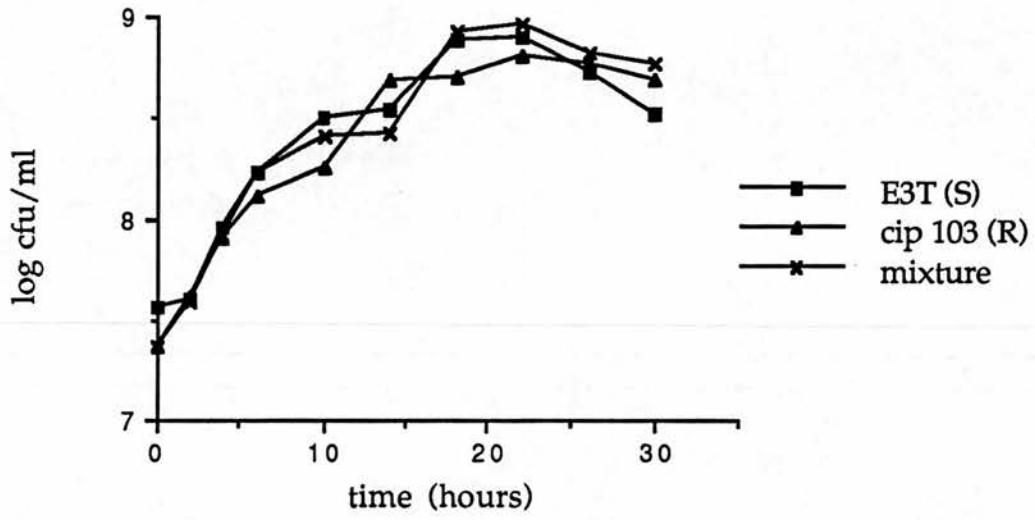


Figure 8. Level of persistence of the ciprofloxacin resistant strain (cip 103) in the mixture of E3T and cip 103

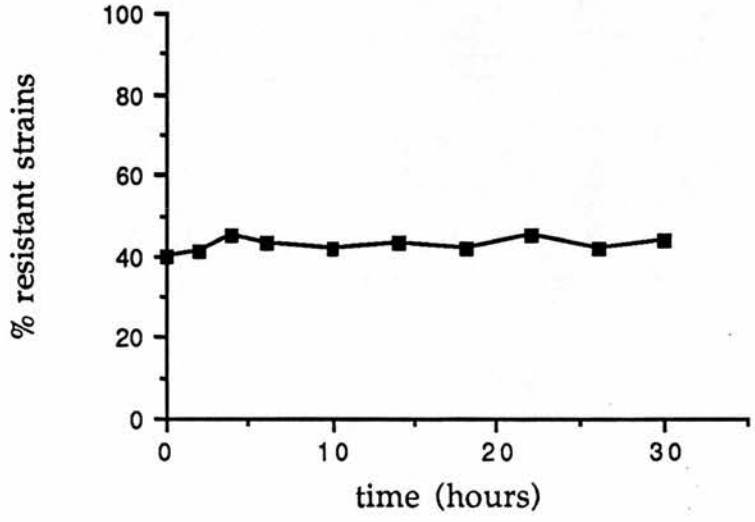


Figure 9. Growth curves of a ciprofloxacin sensitive strain (6989), a ciprofloxacin resistant strain (3225) and a mixture of the two

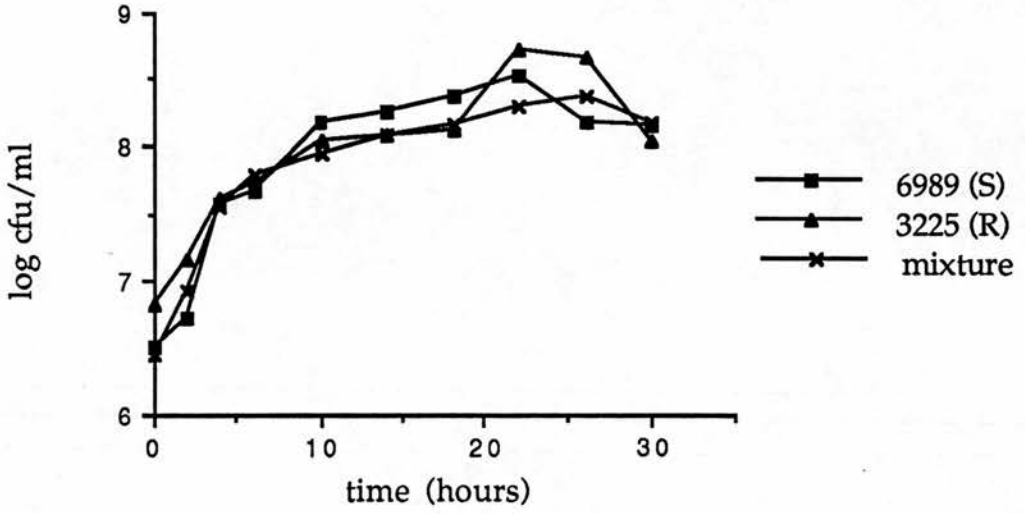


Figure 10. Level of persistence of the ciprofloxacin resistant strain (3225) in the mixture of 6989 and 3225

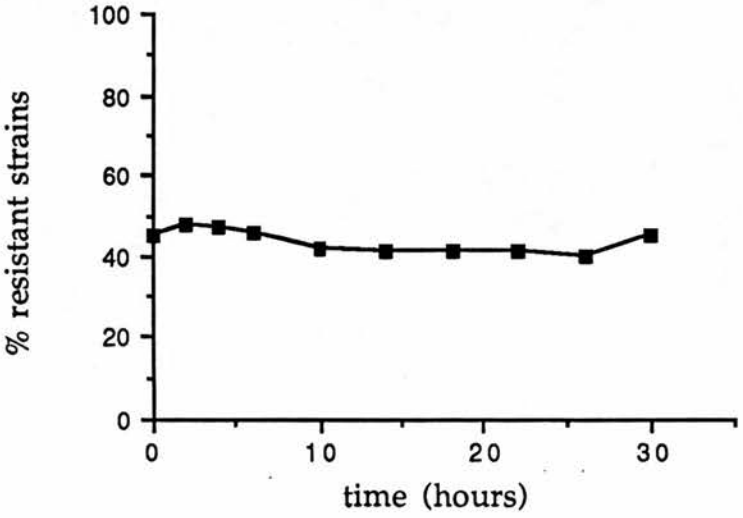


Table 6. Growth of bacterial strains after 18 hours

Strain	Initial inoculum (cfu/ml)	Anaerobic (cfu/ml)	Aerobic (cfu/ml)
E3T	2.5×10^6	1.9×10^8	2.1×10^8
cip 63	1.7×10^6	2.7×10^8	3.1×10^8
cip 86	3.5×10^6	1.2×10^8	1.5×10^8
cip 92	1.8×10^6	3.0×10^8	1.3×10^8
cip 103	1.5×10^6	3.5×10^8	2.5×10^7
cip 132	1.9×10^6	8.0×10^7	3.0×10^8

3.3 Production of Coagulase

3.3.1 Chromogenic assay for Staphylocoagulase

Staphylocoagulase is an extracellular protein which has the ability to stimulate the clotting reaction of plasma, by forming an active molecular complex with prothrombin. The two methods which are accepted for the detection of coagulase, the slide test and the tube test, are prone to false positive and negative reactions. Also, there is difficultly in standardising and quantifying these assays. Therefore another method for detecting coagulase production was devised by Engels *et al* (1981), which is more reliable than the clotting assays. It is a direct assay of Staphylothrombin based on the limited proteolysis of a synthetic chromogenic substrate. The thrombin-like activity of Staphylothrombin cleaves the synthetic tripeptide Chromozym T H, liberating p-nitroanaline, which is yellow.

The previously described method was used to measure soluble

coagulase production only by assaying the supernatant fluid of an overnight culture. Therefore the assay was revised so that it could distinguish soluble and bound coagulase.

3.3.2 Purification of Staphylocoagulase

Staphylocoagulase was purified according to section 2.4.8. The tube coagulase test was used to detect the presence of coagulase during the purification procedure. Coagulase was present in the culture filtrate going into the bovine prothrombin Sepharose 4B column (minimum clotting dose 1: 32), but was not present in the liquid coming off the column, suggesting that it had bound to the column. The first few fractions after elution with 1M NaSCN were tested for the presence of coagulase and the clotting reaction showed that there was no coagulase present. However, it was thought that coagulase was present in the elutant, but that the clotting assay was not working because of the presence of 1M NaSCN, which was interfering with the reaction. The elutants were dialysed overnight in phosphate buffer and the tube coagulase reaction was repeated. This time a clot developed, indicating the presence of coagulase. The purified coagulase had a minimum clotting dose of 1: 2500.

The protein concentration of the purified coagulase was determined as 5.2 mg/ml. A small amount of the coagulase was run on a homogeneous 20% gel by SDS- PAGE using a Phast System (Pharmacia

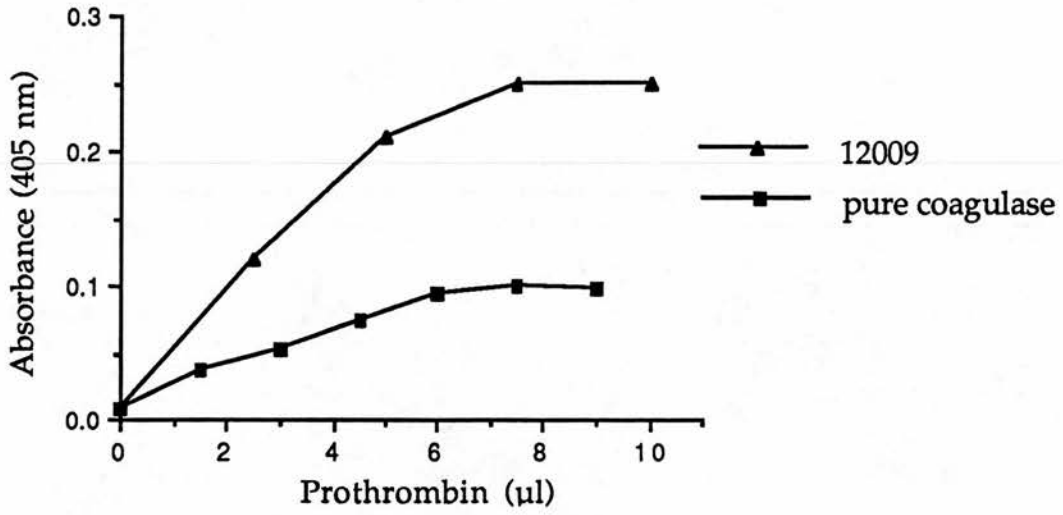
LKB). However, the protein did not show up on the gel, even with silver staining. So it was assumed that the sample was 100 % pure in all subsequent calculations as no contaminating protein could be identified.

3.3.3 Modification of Chromogenic Substrate assay

The purified coagulase was used to develop the chromogenic substrate assay. First the amount of prothrombin needed in the assay was established, since it had been acquired from a different source than the original method of Engels *et al* (1981). Different amounts of prothrombin were used with 2 µl coagulase or 30 µl of an overnight culture of strain 12009, which is known to produce high levels of coagulase, and 123 µl reaction buffer. The absorbance at 405 nm was noted after two hours. Figure 11 shows that the relationship between prothrombin concentration and absorbance is linear up to a certain level of prothrombin and then it is in excess. When pure coagulase was used with the different amounts of prothrombin any amount of prothrombin over 6 µl was in excess. Figure 11 shows that over 8 µl of prothrombin was in excess when strain 12009 was used as the coagulase source. Since strain 12009 is a high producer of coagulase, any amount greater than 8 µl of prothrombin should be in excess for most strains and should not be a limiting factor in the reaction. Therefore, in all subsequent assays 9 µl prothrombin was used.

The purified coagulase was used to construct a standard curve of

Figure 11. Optimisation of prothrombin amounts for chromogenic substrate assay using purified coagulase and strain 12009 as the coagulase sources



coagulase concentrations against time (Figures 12 and 13). Figure 13 would be used in subsequent assays to estimate the amount of coagulase in the reaction mixtures.

Since the original assay measured soluble coagulase production only, the assay was revised so that it measured bound as well as soluble coagulase. An overnight culture of strain 12009, which is known to produce high levels of coagulase, was centrifuged and the supernatant removed. The cell pellet was washed and resuspended in DM. Then this resuspended cell pellet was assayed to estimate the bound coagulase production. The original culture could be used to measure the combined coagulase production. However, an inhibitor would be needed in this assay mixture, since there might be bacterial growth during the two hour incubation period. The inhibitor used was carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which is an inhibitor of cell growth. Ten mls of culture were grown in the presence of different concentrations of inhibitor. Viable counts were taken at the beginning (T= 0) and after two hours (T = 2). The recommended concentration of CCCP was 50 μM but this was found to kill the cells rather than inhibit their growth. Other concentrations were used and the one which was finally used was 5 μM since this inhibited growth without killing the cells (Table 7).

Figure 12. Time course of the hydrolysis of Chromozym TH with different levels of coagulase

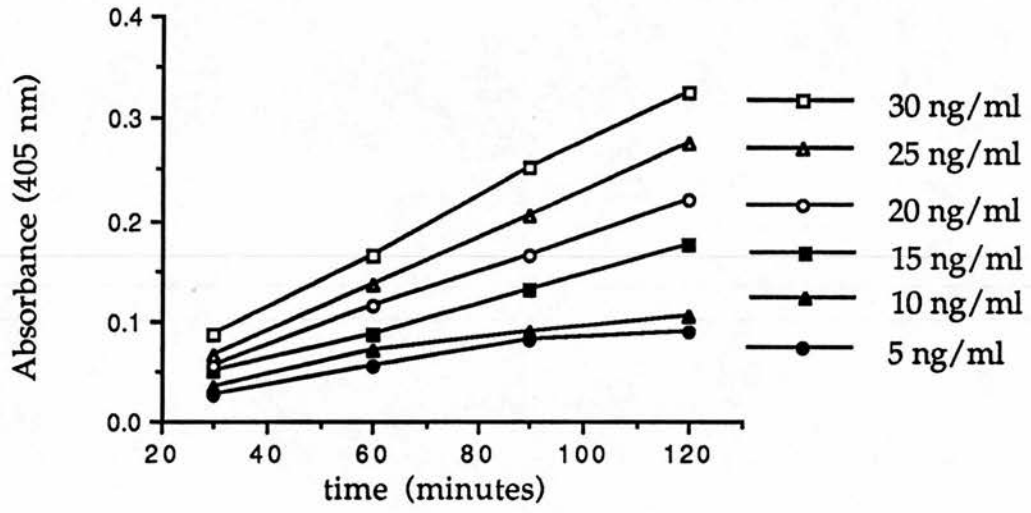


Figure 13. Hydrolysis of Chromozym TH in 2 hours by coagulase

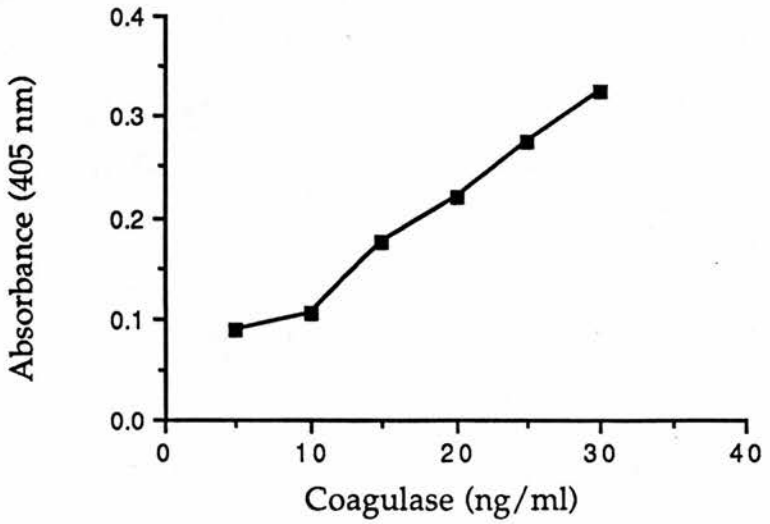


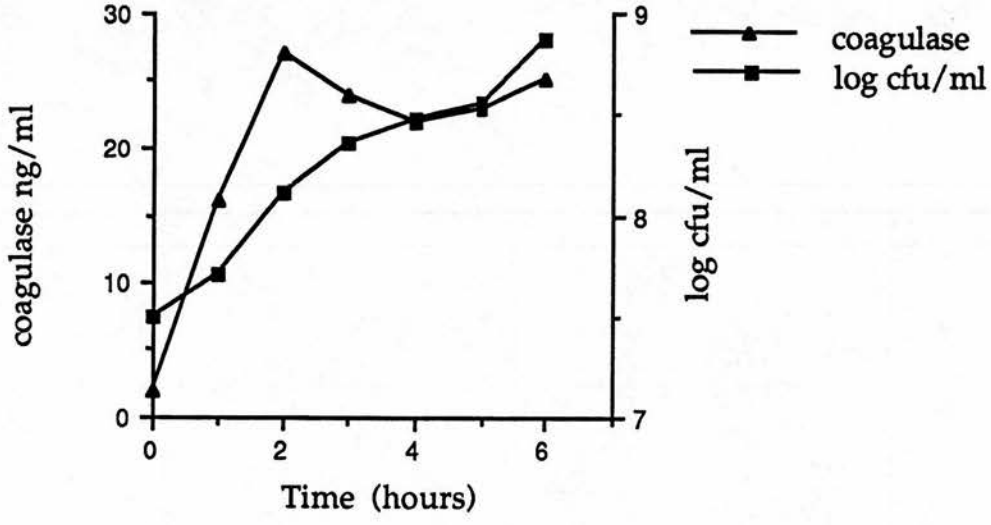
Table 7. Growth (cfu/ml) of strain 12009 in the presence of an inhibitor (CCCP)

Time (hours)	Concentration of CCCP		
	50μM	25μM	5μM
0	3.8×10^8	3.6×10^8	3.9×10^8
2	3.2×10^8	3.3×10^8	4.0×10^8

Coagulase is produced mainly in early exponential phase and it had been noticed in the course of the previous experiments, that if the overnight culture was subcultured into fresh broth, a much higher coagulase reading was obtained. It was therefore decided that the production of coagulase over a period of time would be monitored so that the optimum time for the production of coagulase during the assay could be determined (Figure 14). Since coagulase was mainly produced in the first 2-3 hours, the assay would measure the level of coagulase after two hours growth.

The final reaction mixture and conditions were as follows : 30 μl sample, 15 μl 50 μM carbonyl cyanide m-chlorophenyl hydrazone, 9 μl prothrombin, 100 μl reaction buffer (Engels *et al* 1981)(which included 5 mM EDTA as recommended by Wegrzynowicz *et al* (1980), to eliminate false positive reactions). The strains were grown in Brain Heart Infusion broth (BHI) supplemented with 0.2 % bovine serum albumin (Altenbern

Figure 14. Production of coagulase by strain 12009



1966). Overnight cultures were subcultured into fresh BHI and grown for two hours in an orbital shaker. After two hours, 1 ml of each culture was centrifuged at low speed (5,800 rpm) in a microfuge for 1 minute. The supernatant was removed into an eppendorf tube. The cell pellet was washed and resuspended in 1 ml DM. The whole culture was labelled sample A, the supernatant was labelled sample B and sample C was the resuspended cell pellet. Since sample B did not need the inhibitor because it did not contain cells, the inhibitor was omitted from the reaction mixture for sample B. Each sample was added to two wells of a microtiter plate. To one of the two wells the reaction mixture, described previously, was added. Prothrombin was omitted from the reaction mixture of the remaining well to provide a bacterial background count. The assay mixtures were incubated for two hours and the optical density was measured at 405 nm by a Titertek Multiskan plate reader. This automatically accounts for the blank medium reactions. Subtraction of the OD_{405} value corresponding to the bacterial background count from the test value gave a measure of the change in absorbance as a result of coagulase. This was then related to the standard curve of coagulase concentrations (Figure 13) to get the concentration of coagulase in the test sample.

3.3.4 Production of coagulase by ciprofloxacin sensitive and resistant strains

The modified coagulase assay was used to compare the amount of

coagulase produced by ciprofloxacin sensitive and resistant strains. Strain 12009 was a strain known to produce high levels of coagulase. The other sensitive strains were standard laboratory strains. Varying amounts of bound and soluble coagulase were produced by the ciprofloxacin sensitive and resistant strains (Table 8). The paired strains produced similar amounts. The ciprofloxacin resistant mutant of strain 12009 (12009-3) produced similar amounts of coagulase to strain 12009. Strain E5662, a *S. epidermidis* strain which does not produce any coagulase, was used as a negative control. It is interesting to note that the combined coagulase amount did not equal the sum of the soluble and the bound coagulase amounts, indeed it was always less. Since this is a general trend and not restricted to individual strains, it must be as a result of the assay procedure. Maybe when the cells are being centrifuged and resuspended, coagulase is released from an intracellular pool giving higher readings for samples B and C. However, as the differences were often small, it may just come from biological variation.

3.4 Production of Protein A

Protein A is a cell wall component which is covalently bound to the peptidoglycan of the cell wall. It binds to the Fc portion of immunoglobulins thus preventing phagocytosis of the bacteria. Since most strains secrete about 15-30% of all their protein A (Forsgen 1970), it is necessary to measure both cell bound and extracellular protein A

Table 8. Coagulase production in ciprofloxacin sensitive and resistant strains of *Staphylococcus aureus* (ng coagulase per 10^9 cells)

Strain	Combined	Soluble	Bound
12009 (S)	22.50	17.70	5.20
12009-3 (R)	23.70	18.50	6.10
E3T (S)	7.80	6.45	1.65
Oxford (S)	4.80	3.45	1.55
Cowan 1 (S)	9.00	7.50	2.00
Wood 46 (S)	3.00	2.50	1.00
E5662 (S)	UD	UD	UD
cip 63 (R)	11.85	10.10	2.15
cip 86 (R)	5.70	4.80	1.40
cip 92 (R)	3.60	2.60	1.20
cip 103 (R)	6.40	5.70	1.80
cip 132 (R)	7.80	6.45	2.40
411-87 (R)	13.00	10.00	4.90
417-87 (R)	12.00	9.00	4.00
591-89 (R)	3.00	2.00	1.30
4953-88 (R)	7.00	5.85	1.65
5538-88 (R)	7.95	7.05	1.80
8984-88 (R)	6.45	5.10	1.35
2219 (S)	14.00	11.50	3.00
2221 (R)	14.50	11.00	3.90
2222(R)	13.00	9.00	4.50
6989 (S)	8.00	6.00	2.00
3225 (R)	10.00	7.00	3.50

UD - undetectable R - resistant to cipro S - sensitive to cipro

when measuring protein A production. Exploiting protein A's ability to bind to immunoglobulins allows protein A production to be measured by an enzyme linked immunosorbent assay.

3.4.1 Production of Cell Bound and Extracellular Protein A

Bound and extracellular protein A production of strain Cowan 1 was followed over a period of 6 hours, as well as bacterial growth. Figures 15 and 16 show that it is produced mainly in exponential phase and so production of protein A was measured after 3 hours growth, when it would be most actively produced.

3.4.2 Production of Protein A by ciprofloxacin sensitive and resistant strains

The production of cell bound and extracellular protein A by ciprofloxacin resistant and sensitive strains was compared (Table 9). All strains produced extracellular protein A but at differing levels. Some strains produced 15-30% extracellular protein A and others produced mainly extracellular protein A. This is in keeping with Winbald and Ericson (1973) who observed that methicillin resistant strains produce mainly extracellular protein A. Most of the strains in Table 9 which produced mainly extracellular protein A were methicillin resistant. Strain Wood 46, which is reported not to produce protein A, did produce low levels of cell bound protein A. Again, strain E5662 was used as a negative control since *S. epidermidis* do not produce protein A. Ciprofloxacin resistance

Figure 15. Production of extracellular protein A by strain Cowan 1

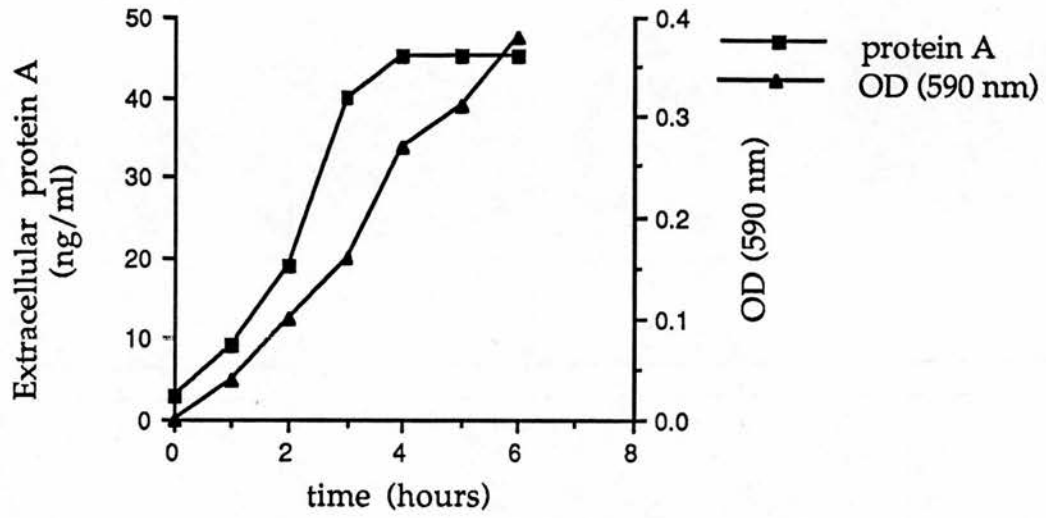


Figure 16. Production of cell bound protein A by strain Cowan 1

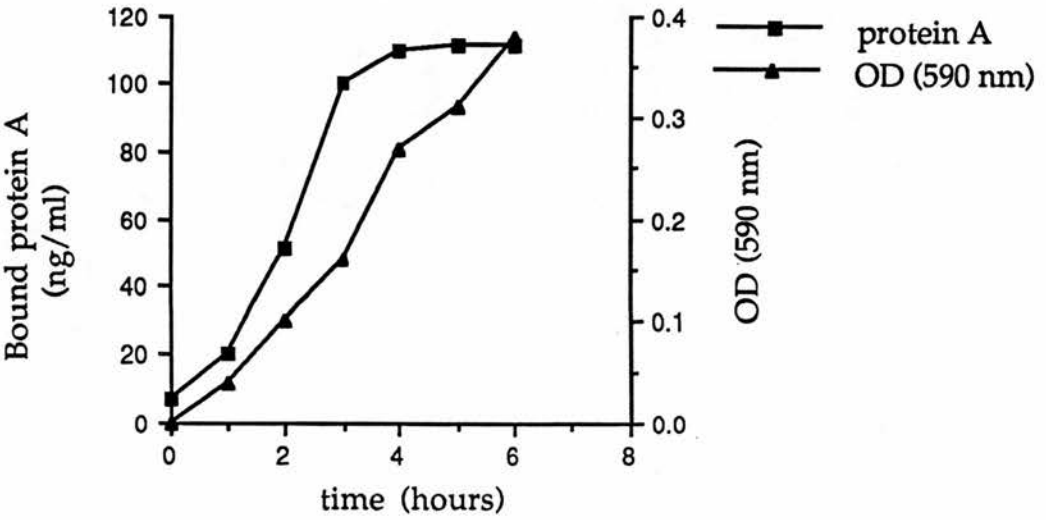


Table 9. Protein A production in ciprofloxacin sensitive and resistant strains of *Staphylococcus aureus* (ng Protein A per mg cell protein)

Strain	Cell Bound	Extracellular
Cowan 1 (S)	79.00	12.00
Cowan 1 - 3 (R)	69.00	10.00
E3T (S)	2.60	0.87
Oxford (S)	0.38	0.09
12009 (S)	3.04	1.05
Wood 46 (S)	0.25	UD
E5662 (S)	UD	UD
cip 63 (R)	60.00	21.00
cip 86 (R)	6.25	3.70
cip 92 (R)	UD	UD
cip 103 (R)	UD	UD
cip 132 (R)	3.40	2.30
411-87 (R)	9.60	1.27
417-87 (R)	0.19	0.06
591-89 (R)	15.00	5.00
4953-88 (R)	8.50	60.00
5538-88 (R)	0.39	40.00
8984-88 (R)	60.00	15.00
2219 (S)	20.00	6.70
2221 (R)	19.00	4.70
2222 (R)	21.00	6.50
6989 (S)	4.80	1.20
3225 (R)	9.80	2.40

UD - undetectable R - resistant to cipro S - sensitive to cipro

did not seem to affect the production of protein A. The sensitive and resistant strains produced similar amounts of protein A. The resistant mutant of Cowan 1 (Cowan 1 - 3) produced similar amounts of protein A to Cowan 1.

3.5 Production of α haemolysin

α haemolysin is thought to be among one of the most significant extracellular proteins produced by *S. aureus*. It is often detected by its haemolytic activity towards erythrocytes but it can also be detected by an ELISA. One disadvantage with this α haemolysin ELISA is the interference of IgG-binding protein A when it is present in the supernatant along with the α haemolysin and other extracellular products. However, this problem was resolved by passing culture fluids through a small adsorption column, which removed all the protein A. All the samples were run on a protein A ELISA to check that all traces of protein A had been removed.

3.5.1 Development of α haemolysin ELISA

An ELISA was developed to detect α haemolysin based on an ELISA for the detection of *S. aureus* delta-like toxins described by Scheifele *et al* (1987). Microtiter plates were coated with two different concentrations of purified α haemolysin which were serially diluted and left at room temperature overnight. The next day the plates were washed with PBS-T

and filled with two different dilutions of α haemolysin antisera. The plates were incubated at room temperature for 90 minutes before being washed again with PBS-T. Enzyme conjugated antibody was added at a concentration recommended by the manufacturer (1:4000) and the plates were incubated at room temperature for 90 minutes. Finally the substrate was added, again at a concentration recommended by the manufacturer (1 mg/ml). The plates were incubated at room temperature for 120 minutes before the absorbance at 405 nm was read on a Titretek Multiskan plate reader. The assay was repeated but with incubation steps at 37°C instead of room temperature.

The optimal conditions for the ELISA were found to be 1:100 dilution of antisera with 250U/ml of α haemolysin as the highest dilution in the serial dilution steps at room temperature. Even though the higher amount of α haemolysin gave higher readings, these readings were at the top end of the absorbance scale at 405 nm (Table 10). Therefore it was decided that the lower concentration of α haemolysin was sufficient for the ELISA. This dilution of antisera (1:100) was used for a 'test' ELISA employing the supernatant of Wood 46 culture as the α haemolysin source. The absorbances after two hours were high and showed good correlation to dilutions of the supernatant. Therefore in all subsequent ELISAs, antisera was used at 1:100 dilution, enzyme conjugated antibody was used at 1:4000 dilution and the substrate used at 1 mg/ml. The ELISA was performed at room temperature. Pure α haemolysin (Gibco) was

used at a starting concentration of 250 U/ml and was serially diluted to give a range of concentrations.

Table 10. Optimal conditions for α haemolysin ELISA (OD $_{405}$ nm)

Anti sera dilutions	Room Temperature α haemolysin 50 µl	10 µl	37°C α haemolysin 50 µl	10 µl
1:100	1.529	0.616	0.647	0.413
1:200	0.389	0.231	0.300	0.212

3.5.2 Production of α haemolysin

The production of α haemolysin by strain Wood 46 was followed over a period of 8 hours. It was produced mainly in late exponential phase and early stationary phase (Figure 17). It was produced after a short lag period of 2 hours, which is a similar finding to Duncan and Cho (1971) who reported a lag period of 3 hours before α haemolysin production commenced. From these results, it was decided that the strains would be grown for five hours before being harvested and measured for α haemolysin production.

3.5.3 Production of α haemolysin by ciprofloxacin sensitive and resistant strains

Production of α haemolysin was measured in ciprofloxacin sensitive and resistant strains. The amount of α haemolysin produced was related to viable cells (Table 11). There seem to be no detectable differences

Figure 17. Production of alpha haemolysin by strain Wood 46

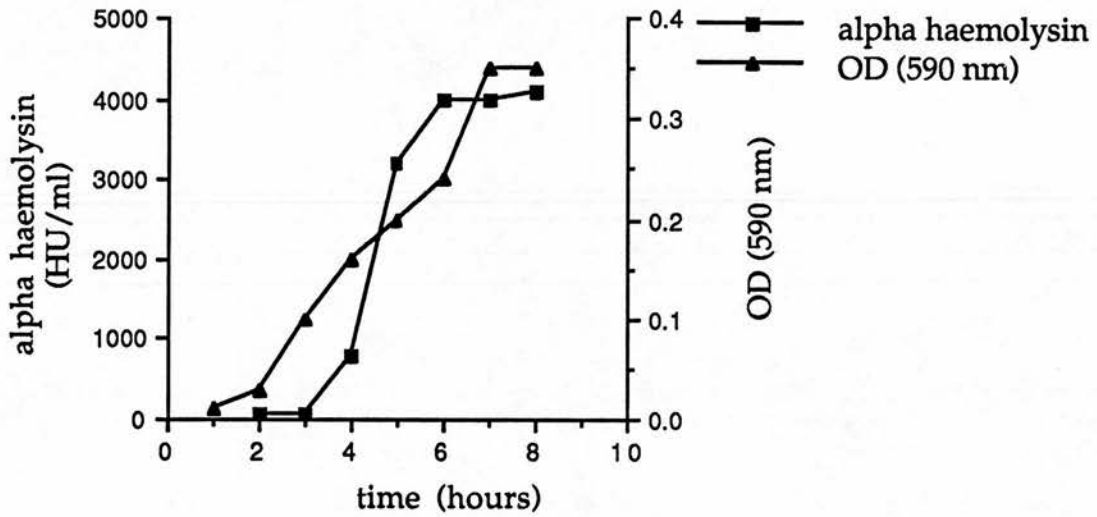


Table 11. α haemolysin production in ciprofloxacin resistant and sensitive strains of *Staphylococcus aureus* (HU/10^9 cells)

Strain	α-haemolysin
Wood 46 (S)	2100
Wood 46-3 (R)	1900
12009 (S)	1300
Cowan 1(S)	48
E3T (S)	77
Oxford (S)	56
cip 63 (R)	59
cip 86 (R)	210
cip 92 (R)	630
cip 103 (R)	40
cip 132 (R)	69
411-87 (R)	56
417-87 (R)	13
591-89 (R)	44
4953-88 (R)	38
5538-88 (R)	15
8984-88 (R)	72
6989 (S)	25
3225 (R)	17
2219 (S)	UD
2221 (R)	UD
2222 (R)	UD

UD - Undetectable (R) - resistant to cipro (S) - sensitive to cipro

between the sensitive strains and the resistant strains. The ciprofloxacin resistant mutant of Wood 46 (Wood 46 -3) produced similar amounts of α haemolysin to Wood 46.

3.6 Production of δ haemolysin

δ haemolysin is another haemolysin which is often detected by its haemolytic activity towards human erythrocytes but can be detected by an ELISA. Again this ELISA is affected by the interference of protein A and this problem is resolved in a similar way as α haemolysin.

3.6.1 Purification of δ haemolysin

δ haemolysin was purified according to section 2.4.13. The final precipitates were dried under vacuum overnight. The haemolytic activity of the precipitates was calculated, to check that they did contain δ haemolysin.

The haemolytic activity of the purified toxin in the precipitates was calculated by the method described in the Section 2.4. 13. The absorption spectrum of haemoglobin was measured, to find the maximum wavelength which was known to be in the 540 nm range. It was found to be 545 nm and so this wavelength was used in all subsequent calculations. 100% haemolysis of the red blood cells was achieved with purified water in the assay mix. Then, varying amounts of the purified

toxin were added to the assay mix, and the OD 545 nm measured. The amount of toxin yielding 50% haemolysis (equivalent to OD $_{545\ nm}$ 0.240) (Figure 18) was taken to represent 1 haemolytic unit. The haemolytic activity of the sample was estimated to be 588 U/ml, since 1.7 µl pure haemolysin caused 50% haemolysis (Figure 19).

The first step in the purification procedure, which involved heating the supernatant to 60°C, should have destroyed any α haemolysin present. However, the purified δ haemolysin was run on an α haemolysin ELISA to check that it did not contain any α haemolysin, which it did not.

3.6.2 Production of δ haemolysin

The production of δ haemolysin by strain E5662 was followed over a period of 8 hours. It was produced mainly in late exponential phase and early stationary phase (Figure 20). From these results, it was decided that the strains would be grown for six hours before being harvested and measured of δ haemolysin production.

3.6.3 Production of δ haemolysin by ciprofloxacin sensitive and resistant strains

δ haemolysin production was investigated in ciprofloxacin sensitive and resistant strains of *S. aureus*. The amount of δ haemolysin produced was related to viable cells (Table 12). There seemed to be no detectable differences between the sensitive and resistant strains. Strain E5662-3,

Figure 18. Estimation of 50% haemolysis in human erythrocytes

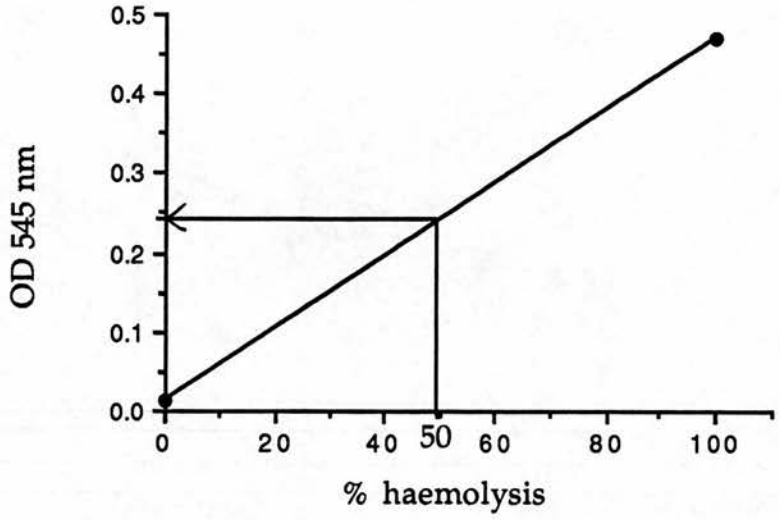


Figure 19. Estimation of the haemolytic activity of purified δ haemolysin

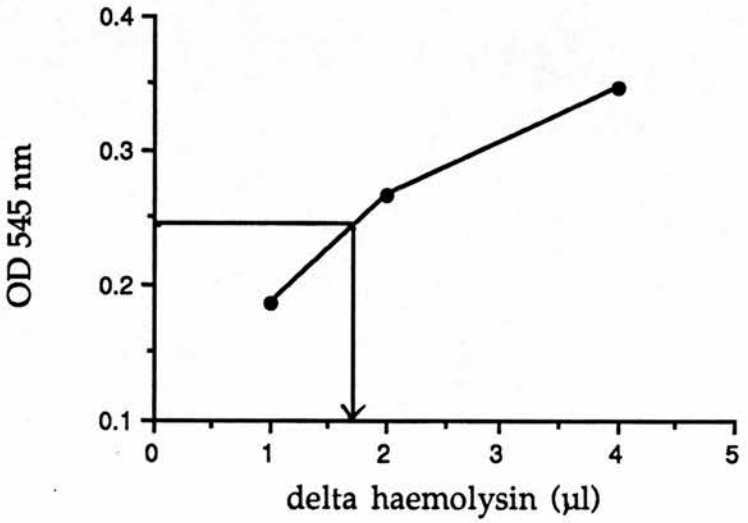


Figure 20. δ haemolysin production by strain E5662

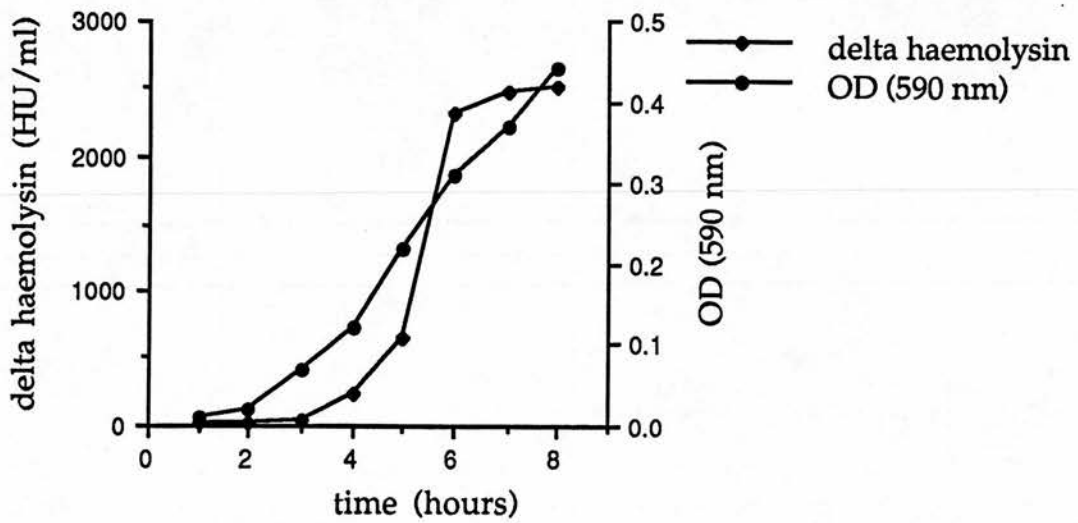


Table 12. δ haemolysin production in ciprofloxacin resistant and sensitive strains of *Staphylococcus aureus* (HU/10^9 cells)

Strain	δ-haemolysin
E5662 (S)	149.0
E5662 - 3 (R)	139.0
E3T (S)	92.0
Oxford (S)	6.2
12009 (S)	52.0
Cowan 1(S)	1.0
Wood 46 (S)	14.0
cip 63 (R)	100.0
cip 86 (R)	70.0
cip 96 (R)	105.0
cip 103 (R)	1.6
cip 132 (R)	55.0
411-87 (R)	10.0
417-87 (R)	21.0
591-89 (R)	UD
4953-88 (R)	UD
5538-88 (R)	0.5
8984-88 (R)	6.0
2219 (S)	UD
2221 (R)	UD
2222 (R)	UD
6989 (S)	56.0
3225 (R)	46.0

UD - undetectable (R) - resistant to cipro (S) - sensitive to cipro

which is a ciprofloxacin resistant mutant of E5662, produced similar amounts of δ haemolysin to the ciprofloxacin sensitive parental strain. The triplet of strains (2219, 2221 and 2222) did not seem to produce any detectable levels of δ haemolysin.

3.7 Pathogenicity of strains *in vivo*

The pathogenicity of *S. aureus* strains *in vivo* was investigated by subcutaneous injection of bacteria into mice. The development of the abscesses was noted over 10 days and any morphological details noted. Mice were also injected with just the cytodex microcarriers - as controls, to check that the inoculum without the test strain was sterile and that the injection procedure was aseptic. Mice injected with just cytodex developed small raised areas which did not develop into abscesses. Their size remained static over the 10 day period. Mice injected with the bacterial inoculum developed abscesses which fluctuated in size and exhibited tissue damage. In some cases a white area covering the skin was seen which developed into necrosis. As the abscesses developed, scabs were also formed.

3.7.1 *In vivo* growth of ciprofloxacin sensitive and resistant strains

When ciprofloxacin sensitive and resistant strains were injected into mice, no differences were observed in the abscesses which developed (Figures 21 and 22). The abscesses looked similar, developed at a similar rate and the degree of tissue damage looked similar.

Figure 21. Development of abscesses by paired isolates of ciprofloxacin sensitive and resistant strains

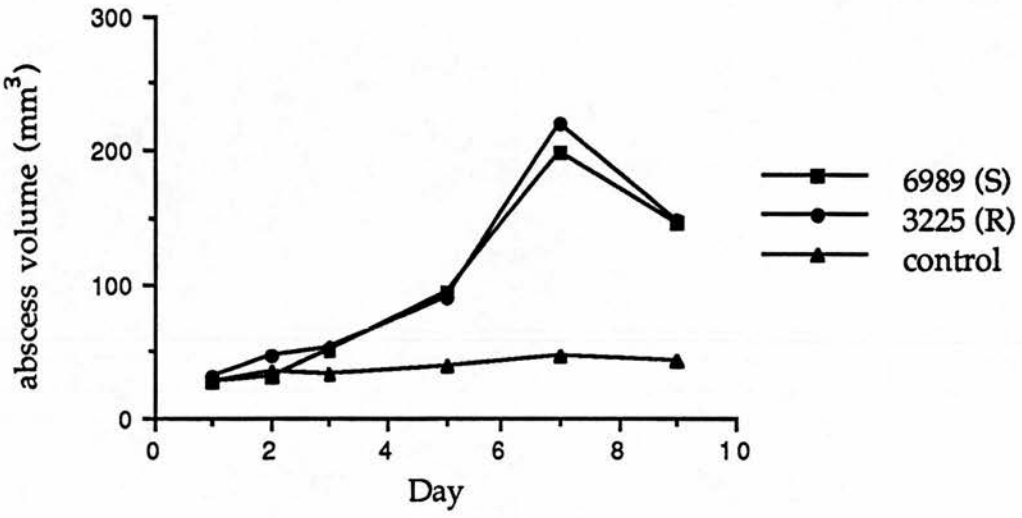
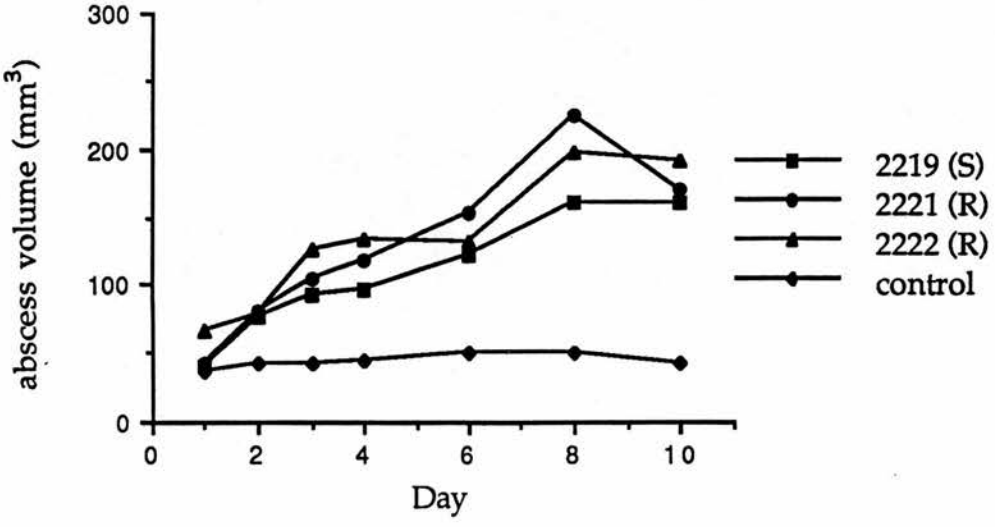


Figure 22. Development of abscesses by triplet of ciprofloxacin sensitive and resistant strains



3.7.2 *In vivo* growth of high virulence factor producing strains

The 4 high factor producing strains were also put into mice. The strain which produces high levels of coagulase produced large abscesses with areas of necrosis. The other 3 strains produced rather small abscesses in comparison with no necrosis and little tissue damage (Figure 23). Two strains were chosen, one which was found to produce high levels of most of the factors studied (strain 8984-88) and one which produced low levels of the factors studied (Oxford Staph). These strains were injected into mice to see if there were any differences in their pathogenicity. Strain 8984-88 produced fairly large abscesses when compared to Oxford Staph (Figure 24). No sign of any necrosis was seen with either strain. Oxford Staph is used extensively in laboratories and has probably been passaged numerous times, which might account for its reduced virulence.

3. 8 Summary

This section investigated the effect of ciprofloxacin resistance on the pathogenicity of some *S. aureus* strains. Ciprofloxacin sensitive and resistant strains were grown statically and with aeration to see if there were any differences in their growth rates. There appeared to be no differences either when grown statically or with aeration. When a ciprofloxacin sensitive strain and a resistant strain were grown in a mixed culture, the ciprofloxacin resistant strains persisted in the

Figure 23. Development of abscesses by high virulence factor producing strains

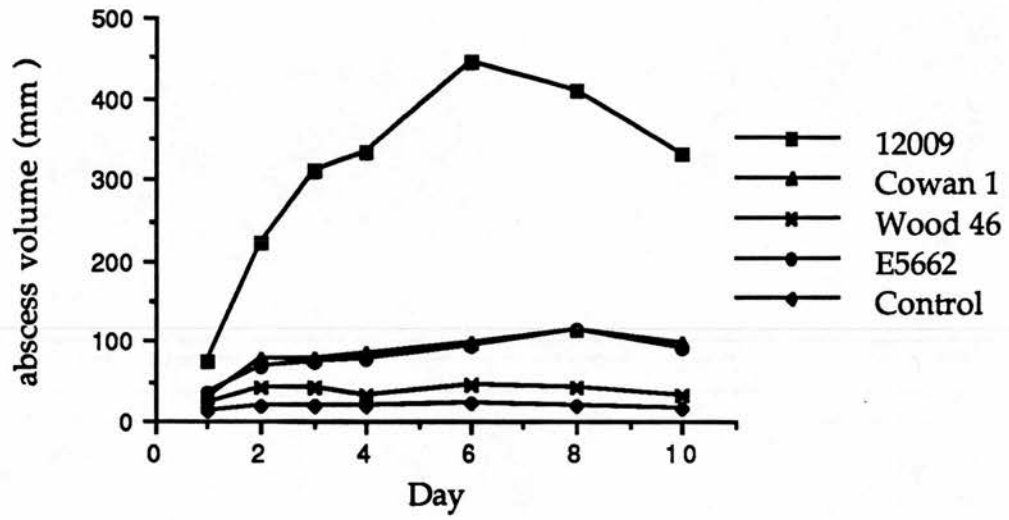
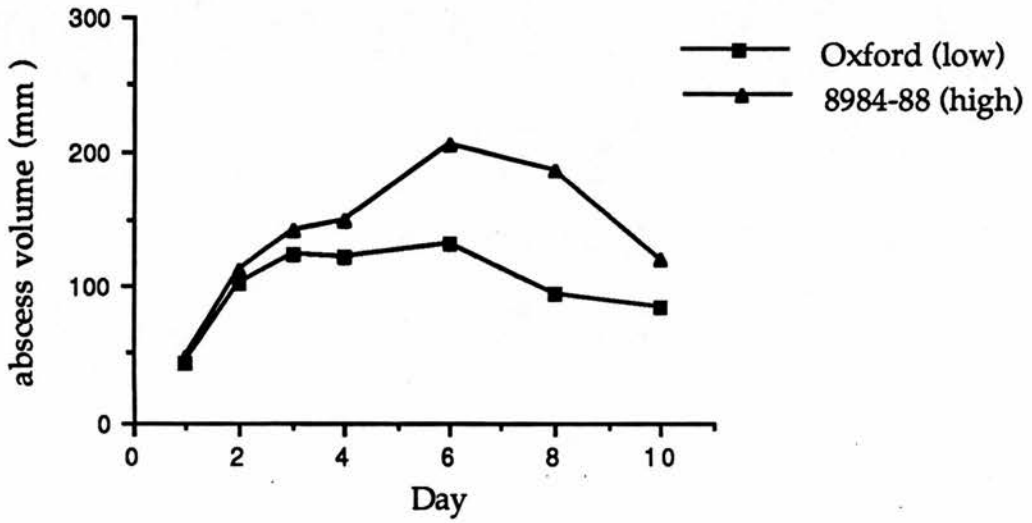


Figure 24. Development of abscesses by a high (8984-88) and a low (Oxford) factor producing strain of the factors studied



mixture. The resistant strains were still able to grow anaerobically, despite reports that resistance sometimes leads to aerobic growth only. Therefore the resistant strains seemed to grow under similar conditions to the sensitive strains.

The ciprofloxacin sensitive and resistant strains were then investigated for the production of four virulence factors - coagulase, protein A, α haemolysin and δ haemolysin. The resistant strains produced similar amounts of these four factors to the sensitive strains. Again there seemed to be no differences between the strains.

Finally, to confirm the findings of the *in vitro* work, some ciprofloxacin sensitive and resistant strains were injected into mice. The behaviour of the strains *in vivo* would confirm or deny if there were any differences between the strains. Again, the resistant strains produced similar sized abscesses to the sensitive strains, so supporting the *in vitro* determinations that there were no differences in the pathogenicity of the resistant strains.

4 Results - Pathogenicity and Sub MIC levels of antibiotics

One of the many ways of investigating pathogenicity is to look at the effect of antibiotics on virulence factor production. Concentrations of antibiotics are used which do not affect cell growth - sub-minimum inhibitory concentrations (sub - MIC). This approach will be employed in the next section. Six antibiotics were investigated for their effect on toxin production :- two quinolones - ciprofloxacin and enoxacin, three protein synthesis inhibitors - gentamicin, chloramphenicol and tetracycline and a cell wall synthesis inhibitor - methicillin. By using antibiotics with different modes of action, a wide variety of effects on virulence factor production should be observed.

4.1 Production of coagulase in the presence of sub-MIC levels of antibiotics

The production of coagulase was monitored in the presence of 1/4 and 1/2 MIC levels of six antibiotics - ciprofloxacin, enoxacin, gentamicin, chloramphenicol, tetracycline and methicillin. The strains were grown overnight as described in section 3.3.3 and then they were subcultured into medium containing the antibiotics. Strains were also grown in broth lacking the antibiotics, so that the production of coagulase under normal conditions could be monitored. The strains were grown for two hours before harvesting and measured for the production of coagulase.

The amount of coagulase produced in the presence of the antibiotics was compared to the amount produced when no antibiotic was present. Viable counts and the total cell protein were also measured for each of the strains, so that coagulase production could be related to these. Since coagulase is predominately an extracellular enzyme, the figures for coagulase production related to viable counts will be discussed here.

Strains grown in the presence of 1/4 and 1/2 MIC levels of the two quinolones, ciprofloxacin and enoxacin, produced similar amounts, within 15%, of coagulase to that produced in the absence of antibiotics (Tables 13, 14 and appendix Tables I and II). These antibiotics did not seem to affect the production of bound or soluble coagulase. The presence of 1/2 or 1/4 MIC levels of methicillin also had little or no effect on coagulase production (Table 15 and appendix table III). The presence of gentamicin, chloramphenicol and tetracycline reduced the production of coagulase to varying degrees (Tables 16 - 21). The effect of 1/2 MIC levels of these three antibiotics was greater than the effect of 1/4 MIC levels. When coagulase production was related to viable bacteria, 1/2 MIC levels of the three antibiotics reduced its production to about 50% of that in the absence of the antibiotics (Tables 16, 18 and 20). Some strains were not affected as much as other strains. When the strains were grown in the presence of 1/4 MIC levels of these three antibiotics, coagulase production was reduced but to a lesser extent - only to 80 - 90 % of that in the absence of the antibiotics (Tables 17, 19 and 21). This

Table 13. The effect of 1/2 MIC levels of ciprofloxacin on the production of coagulase

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% of amount produced in absence	ng coagulase/ 10^9 cells	% of amount produced in absence
Sensitive bacteria				
12009	22.73	101	6.97	101
E3T	4.69	102	1.39	99
Oxford	6.86	104	3.07	99
Cowan 1	8.82	98	0.95	106
Wood 46	2.30	92	0.86	95
Resistant bacteria				
cip 63	15.51	110	5.30	106
cip 86	4.75	99	1.14	104
cip 92	2.76	106	1.05	105
cip 103	5.61	102	1.56	104
cip 132	4.55	101	0.61	102
411-87	10.40	104	3.09	103
417-87	9.22	97	2.38	99
591-89	1.90	95	1.39	99
4953-88	5.20	104	1.12	101
5538-88	6.83	102	1.41	101
8984-88	3.85	110	0.98	109
Triplet				
2219	11.86	104	3.18	106
2221	12.10	110	4.24	106
2222	9.11	99	4.09	95
Paired strains				
6989	5.89	95	1.46	97
3225	6.77	101	3.43	98

Table 14. The effect of 1/2 MIC levels of enoxacin on the production of coagulase

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% of amount produced in absence	ng coagulase/ 10^9 cells	% of amount produced in absence
Sensitive bacteria				
12009	23.40	104	6.83	99
E3T	4.69	102	1.44	103
Oxford	6.67	101	3.16	102
Cowan 1	9.36	104	0.95	106
Wood 46	2.45	98	0.94	104
Resistant bacteria				
cip 63	14.24	101	4.95	99
cip 86	4.90	102	1.13	103
cip 92	2.76	102	1.04	104
cip 103	5.67	103	1.53	102
cip 132	4.77	106	0.61	102
411-87	10.40	104	3.21	107
417-87	9.69	102	2.50	104
591-89	2.02	101	1.44	103
4953-88	5.10	102	1.17	104
5538-88	7.17	107	1.44	103
8984-88	3.64	104	0.92	102
Triplet				
2219	12.08	106	3.15	105
2221	11.20	102	4.16	104
2222	9.29	101	4.43	103
Paired strains				
6989	6.32	102	1.52	101
3225	6.83	102	3.54	101

Table 15. The effect of 1/2 MIC levels of methicillin on the production of coagulase

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% of amount produced in absence	ng coagulase/ 10^9 cells	% of amount produced in absence
Sensitive bacteria				
12009	24.53	109	7.45	108
E3T	4.92	107	1.53	109
Oxford	6.93	105	3.32	107
Cowan 1	9.36	104	0.94	104
Wood 46	2.73	109	0.91	101
Resistant bacteria				
cip 63	13.96	99	5.30	106
cip 86	4.90	102	1.14	104
cip 92	2.68	103	1.02	102
cip 103	5.56	101	1.56	104
cip 132	4.64	103	0.61	102
411-87	10.40	104	3.06	102
417-87	9.88	104	2.47	103
591-89	2.08	104	1.43	102
4953-88	5.05	101	1.12	102
5538-88	6.97	104	1.53	109
8984-88	3.78	108	0.96	107
Triplet				
2219	12.20	107	3.18	106
2221	11.55	105	4.16	104
2222	9.66	105	4.43	101
Paired strains				
6989	6.63	107	1.64	109
3225	6.77	101	3.57	102

Table 16. The effect of 1/2 MIC levels of gentamicin on the production of coagulase

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% of amount produced in absence	ng coagulase/ 10^9 cells	% of amount produced in absence
Sensitive bacteria				
12009	11.70	52	3.74	54
E3T	2.71	59	0.66	47
Oxford	2.77	42	1.52	49
Cowan 1	4.59	51	0.50	55
Wood 46	1.40	56	0.51	57
Resistant bacteria				
cip 63	8.32	59	3.05	61
cip 86	2.98	62	0.70	64
cip 92	1.43	55	0.61	61
cip 103	3.30	60	0.86	57
cip 132	2.48	55	0.29	49
411-87	4.30	43	1.41	47
417-87	4.85	51	1.18	49
591-89	0.90	45	0.67	48
4953-88	2.35	47	0.48	44
5538-88	3.42	51	0.69	49
8984-88	2.00	57	0.50	55
Triplet				
2219	5.93	52	1.62	54
2221	5.39	49	2.16	54
2222	5.43	59	2.24	52
Paired strains				
6989	3.16	51	0.80	53
3225	3.28	49	1.82	52

Table 17. The effect of 1/4 MIC levels of gentamicin on coagulase production

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% of amount produced in absence	ng coagulase/ 10^9 cells	% of amount produced in absence
Sensitive bacteria				
12009	18.90	84	6.00	84
E3T	3.77	82	1.22	87
Oxford	6.01	91	2.79	90
Cowan 1	7.47	83	0.72	80
Wood 46	2.03	81	0.78	87
Resistant bacteria				
cip 63	11.14	79	4.15	83
cip 86	4.56	95	0.99	90
cip 92	2.00	77	0.75	75
cip 103	4.51	82	1.28	85
cip 132	4.05	90	0.49	82
411-87	7.90	79	2.46	82
417-87	7.98	84	2.04	85
591-89	1.58	79	1.01	72
4953-88	4.70	94	1.01	92
5538-88	5.96	89	1.26	90
8984-88	2.87	82	0.78	87
Triplet				
2219	9.80	86	2.43	81
2221	8.69	79	3.00	75
2222	7.45	81	3.57	83
Paired strains				
6989	5.27	85	1.31	87
3225	5.29	79	2.84	81

Table 18. The effect of 1/2 MIC levels of chloramphenicol on the production of coagulase

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% of amount produced in absence	ng coagulase/ 10^9 cells	% of amount produced in absence
Sensitive bacteria				
12009	11.70	52	3.73	54
E3T	2.44	53	0.71	51
Oxford	3.10	47	1.58	51
Cowan 1	4.68	52	0.49	54
Wood 46	1.23	49	0.42	47
Resistant bacteria				
cip 63	13.82	98	5.05	101
cip 86	2.83	59	0.67	61
cip 92	1.22	47	0.51	51
cip 103	2.31	42	0.62	41
cip 132	1.76	39	0.25	41
411-87	4.30	43	1.32	44
417-87	5.89	62	1.51	63
591-89	1.18	59	0.77	55
4953-88	2.55	51	0.52	47
5538-88	3.28	49	0.57	41
8984-88	1.65	47	0.47	52
Triplet				
2219	6.27	55	1.83	61
2221	6.27	57	2.04	51
2222	4.97	54	2.37	55
Paired strains				
6989	4.28	69	0.89	59
3225	3.15	47	1.79	51

Table 19. The effect of 1/4 MIC levels of chloramphenicol on the production of coagulase

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% of amount produced in absence	ng coagulase/ 10^9 cells	% of amount produced in absence
Sensitive bacteria				
12009	20.48	91	6.14	89
E3T	3.73	81	1.16	83
Oxford	5.54	84	2.70	87
Cowan 1	8.19	91	0.83	92
Wood 46	2.10	84	0.78	87
Resistant bacteria				
cip 63	13.25	94	4.95	99
cip 86	3.94	82	0.92	84
cip 92	2.42	93	0.90	90
cip 103	5.45	99	1.52	101
cip 132	3.69	82	0.50	84
411-87	8.20	82	2.37	79
417-87	7.79	82	1.80	75
591-89	1.48	74	1.19	85
4953-88	4.10	82	0.92	84
5538-88	5.90	88	1.27	91
8984-88	3.15	90	0.73	81
Triplet				
2219	9.35	82	2.52	84
2221	9.57	87	3.56	89
2222	7.54	82	3.83	89
Paired strains				
6989	5.64	91	1.41	94
3225	6.16	92	3.26	93

Table 20. The effect of 1/2 MIC levels of tetracycline on the production of coagulase

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% of amount produced in absence	ng coagulase/ 10^9 cells	% of amount produced in absence
Sensitive bacteria				
12009	10.58	47	2.90	42
E3T	2.35	51	0.69	54
Oxford	3.63	55	1.64	53
Cowan 1	4.41	49	0.49	49
Wood 46	1.15	46	0.38	42
Resistant bacteria				
cip 63	14.24	101	4.85	97
cip 86	2.02	42	0.46	42
cip 92	1.53	59	0.47	47
cip 103	3.03	55	0.77	51
cip 132	2.12	47	0.31	51
411-87	5.90	59	1.74	58
417-87	4.37	46	0.98	41
591-89	0.86	43	0.69	49
4953-88	2.55	51	0.61	55
5538-88	6.83	102	1.41	101
8984-88	1.65	47	0.50	55
Triplet				
2219	6.50	57	1.62	54
2221	5.61	51	2.12	53
2222	3.77	41	1.85	43
Paired strains				
6989	2.60	42	0.74	49
3225	3.42	51	1.61	46

Table 21. The effect of 1/4 MIC levels of tetracycline on the production of coagulase

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% of amount produced in absence	ng coagulase/ 10^9 cells	% of amount produced in absence
Sensitive bacteria				
12009	18.45	82	5.87	85
E3T	4.23	92	1.36	97
Oxford	5.35	81	2.60	84
Cowan 1	8.46	94	0.87	97
Wood 46	2.03	81	0.80	89
Resistant bacteria				
cip 63	12.27	87	4.10	82
cip 86	4.37	91	1.02	93
cip 92	2.13	82	0.87	87
cip 103	4.51	82	1.25	83
cip 132	4.01	89	0.52	87
411-87	7.20	72	2.20	74
417-87	7.51	79	1.94	81
591-89	1.64	82	1.18	84
4953-88	4.35	87	0.98	89
5538-88	5.63	84	1.16	83
8984-88	3.19	91	0.86	95
Triplet				
2219	10.49	92	2.70	90
2221	9.24	84	3.16	79
2222	8.74	95	4.26	99
Paired strains				
6989	5.08	82	1.26	84
3225	5.76	86	3.12	89

suggests that these antibiotics, which are protein synthesis inhibitors, not only affect the production of coagulase but also might affect some other aspect of its production.

Two of the strains behaved differently in the presence of 1/2 MIC levels of chloramphenicol and tetracycline (strains cip 63 and 5538-88). They were not affected by 1/2 MIC levels of tetracycline and cip 63 was not affected by 1/2 MIC levels of chloramphenicol. Coagulase was produced in similar amounts in the presence of these antibiotics to that produced in the absence of these antibiotics. These two strains were highly resistant to tetracycline and so it could have been related to the resistance mechanism to tetracycline.

4.2 Production of Protein A in the presence of sub-MIC levels of antibiotics

Protein A production was monitored in the presence of the same six antibiotics. The strains were grown overnight and then subcultured into fresh broth. They were grown for an hour, the antibiotics were then added and growth continued for a further two hours. Strains were also grown in the absence of the antibiotics for a comparison. The strains were harvested and measured for cell bound and extracellular protein A production and total cell protein. The amount of protein A produced in the presence of the antibiotics was compared to the amount produced in

the absence.

The presence of ciprofloxacin, enoxacin and methicillin at 1/2 or 1/4 MIC levels had little or no effect on the production of either bound or extracellular protein A (Tables 22 - 27). The amount of protein A produced in the presence of these antibiotics was similar to the amount produced in the absence. The effect of 1/2 and 1/4 MIC levels of gentamicin had a limited effect on protein A production (Table 28 and 29). At 1/2 MIC levels the production of protein A was reduced by varying amounts. In some strains it was reduced to 40 - 60 % of that produced in the absence of the antibiotics. In other strains the effect was not so pronounced and they produced 80 - 90 % of the normal amounts of protein A. The other two protein synthesis inhibitors reduced the production of protein A considerably more (Tables 30 - 33). At 1/2 MIC levels, the production of protein A was greatly reduced, to only a fraction of that in the absence of the antibiotics. In some cases, the level of cell bound and especially extracellular protein A was reduced to undetectable levels. At 1/4 MIC levels these two antibiotics still reduced protein A production but to a lesser extent. Most strains produced about 80% of the protein A found in the absence of antibiotics. Other strains were either affected to a greater extent or not affected at all. The effect of gentamicin, chloramphenicol and tetracycline on bound versus extracellular protein A was varied. In most strains the production of extracellular protein A was reduced more than bound protein A, but in a few strains the

Table 22. The effect of 1/4 MIC levels of ciprofloxacin on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	59.00	98	7.60	104
E3T	0.75	104	0.37	93
Oxford	0.44	94	0.30	103
12009	1.32	97	0.80	104
Wood 46	0.45	100	UD	–
Resistant bacteria				
cip 63	46.00	105	13.30	112
cip 86	1.73	113	0.73	102
cip 92	UD	–	UD	–
cip 103	UD	–	UD	–
cip 132	3.70	105	1.00	91
411-87	29.00	97	15.40	101
417-87	0.18	100	0.05	92
591-89	24.90	99	10.25	95
4953-88	2.57	89	35.60	102
5538-88	3.50	88	44.20	98
8984-88	10.17	102	32.30	92
Triplet				
2219	27.00	100	9.40	94
2221	25.00	108	12.50	104
2222	23.00	96	11.00	102
Paired strains				
6989	28.00	93	5.98	101
3225	37.00	106	12.10	94

UD - undetectable Sp A - Protein A

Table 23. The effect of 1/2 MIC levels of ciprofloxacin on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	75.00	95	11.00	100
E3T	2.45	94	0.88	101
Oxford	0.35	92	0.10	94
12009	3.12	103	1.08	99
Wood 46	0.25	100	UD	–
Resistant bacteria				
cip 63	59.30	99	20.10	95
cip 86	6.00	97	3.65	99
cip 92	UD	–	UD	–
cip 103	UD	–	UD	–
cip 132	4.10	121	2.20	96
411-87	9.70	101	1.30	102
417-87	0.19	97	0.06	98
591-89	14.30	97	4.30	95
4953-88	8.47	99	58.00	97
5538-88	0.42	107	37.00	93
8984-88	56.00	93	14.00	93
Triplet				
2219	19.00	95	6.30	94
2221	17.00	89	4.80	102
2222	21.00	100	6.80	105
Paired strains				
6989	4.90	102	1.10	92
3225	9.70	97	2.25	94

UD - undetectable Sp A - Protein A

Table 24. The effect of 1/4 MIC levels of enoxacin on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	58.50	97	7.50	97
E3T	0.71	99	0.37	93
Oxford	0.47	100	0.30	105
12009	1.33	98	0.72	93
Wood 46	0.45	100	UD	-
Resistant bacteria				
cip 63	42.00	95	10.90	92
cip 86	1.49	97	0.70	99
cip 92	UD	-	UD	-
cip 103	UD	-	UD	-
cip 132	3.60	103	1.01	91
411-87	29.00	97	15.60	103
417-87	0.17	94	0.06	98
591-89	24.00	96	10.17	94
4953-88	2.54	88	36.80	105
5538-88	4.10	105	43.60	97
8984-88	34.00	97	9.80	98
Triplet				
2219	28.00	104	9.70	97
2221	23.00	100	12.80	107
2222	23.00	96	10.70	99
Paired strains				
6989	27.00	90	5.80	98
3225	35.00	100	13.90	107

UD - undetectable Sp A - Protein A

Table 25. The effect of 1/2 MIC levels of enoxacin on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	74.00	94	10.00	91
E3T	2.80	107	0.79	91
Oxford	0.40	106	0.10	98
12009	2.90	94	1.08	99
Wood 46	0.30	111	UD	-
Resistant bacteria				
cip 63	59.00	99	20.90	99
cip 86	5.90	95	3.64	98
cip 92	UD	-	UD	-
cip 103	UD	-	UD	-
cip 132	3.10	91	2.10	90
411-87	10.00	104	1.16	91
417-87	0.19	97	0.06	100
591-89	13.60	93	43.00	95
4953-88	7.70	91	57.00	95
5538-88	0.39	100	39.00	98
8984-88	55.00	92	14.00	93
Triplet				
2219	18.00	90	6.75	110
2221	18.00	95	4.70	100
2222	22.00	105	6.90	107
Paired strains				
6989	4.80	100	1.12	94
3225	10.10	101	2.40	100

UD - undetectable Sp A - Protein A

Table 26. The effect of 1/4 MIC levels of methicillin on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	59.00	98	7.30	95
E3T	0.67	94	0.38	94
Oxford	0.46	98	0.28	96
12009	1.33	98	0.75	97
Wood 46	0.46	104	UD	–
Resistant bacteria				
cip 63	43.00	98	11.05	99
cip 86	1.68	110	0.70	99
cip 92	UD	–	UD	–
cip 103	UD	–	UD	–
cip 132	3.60	103	1.03	94
411-87	31.00	103	15.30	101
417-87	0.16	88	0.50	89
591-89	25.00	100	9.70	90
4953-88	3.01	104	31.20	89
5538-88	3.40	85	49.80	111
8984-88	35.00	100	9.75	98
Triplet				
2219	26.50	98	11.00	110
2221	23.00	100	12.48	104
2222	21.00	88	9.76	90
Paired strains				
6989	27.00	90	6.40	108
3225	37.00	106	11.40	88

UD - undetectable Sp A - Protein A

Table 27. The effect of 1/2 MIC levels of methicillin on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	77.00	97	10.00	91
E3T	2.90	115	0.53	61
Oxford	0.41	109	0.11	105
12009	2.67	88	1.01	99
Wood 46	0.23	92	UD	-
Resistant bacteria				
cip 63	56.50	95	22.50	106
cip 86	5.80	93	3.40	92
cip 92	UD	-	UD	-
cip 103	UD	-	UD	-
cip 132	3.80	113	2.30	98
411-87	10.70	111	1.26	100
417-87	0.19	98	0.06	100
519-89	11.25	76	4.20	94
4953-88	10.00	113	61.00	102
5538-88	0.16	41	17.00	43
8984-88	64.00	107	15.00	100
Triplet				
2219	19.00	95	6.36	95
2221	18.00	95	4.50	96
2222	20.00	95	7.20	110
Paired strains				
6989	4.80	100	1.15	96
3225	9.25	93	2.30	96

UD - undetectable Sp A - Protein A

Table 28. The effect of 1/4 MIC levels of gentamicin on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	61.00	102	7.20	92
E3T	0.71	98	0.20	50
Oxford	0.36	71	0.32	110
12009	1.30	96	0.62	81
Wood 46	0.35	77	UD	–
Resistant bacteria				
cip 63	41.50	94	11.60	100
cip 86	1.67	109	0.66	94
cip 92	UD	–	UD	–
cip 103	UD	–	UD	–
cip 132	3.30	95	1.07	98
411-87	28.00	93	15.10	99
417-87	0.18	100	0.06	105
591-89	24.00	96	10.50	98
4953-88	2.74	95	33.60	104
5538-88	4.15	104	34.20	77
8984-88	35.50	101	10.25	103
Triplet				
2219	25.50	93	9.70	97
2221	26.00	110	11.70	98
2222	22.00	92	9.70	90
Paired strains				
6989	31.00	103	6.12	104
3225	32.00	91	5.90	46

UD - undetectable Sp A - Protein A

Table 29. The effect of 1/2 MIC levels of gentamicin on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	70.00	89	8.00	73
E3T	1.30	50	0.26	30
Oxford	0.23	60	0.09	85
12009	1.77	58	0.65	62
Wood 46	0.10	40	UD	–
Resistant bacteria				
cip 63	57.70	97	21.40	101
cip 86	7.60	121	3.98	107
cip 92	UD	–	UD	–
cip 103	UD	–	UD	–
cip 132	3.10	94	2.40	104
411-87	10.80	113	1.21	95
417-87	0.18	92	UD	–
591-89	15.50	104	3.20	70
4953-88	6.30	75	58.00	97
5538-88	0.29	74	37.00	93
8984-88	29.00	49	10.00	67
Triplet				
2219	17.00	85	4.28	64
2221	17.00	89	2.50	53
2222	18.00	86	4.80	74
Paired strains				
6989	4.20	80	1.00	80
3225	8.30	83	2.10	89

UD - undetectable Sp A - Protein A

Table 30. The effect of 1/4 MIC levels of chloramphenicol on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	50.00	83	7.00	89
E3T	0.73	101	0.14	36
Oxford	0.33	70	0.12	41
12009	1.15	84	0.45	59
Wood 46	0.30	67	UD	–
Resistant bacteria				
cip 63	39.00	88	9.20	78
cip 86	1.52	99	0.58	82
cip 92	UD	–	UD	–
cip 103	UD	–	UD	–
cip 132	2.80	80	1.23	112
411-87	27.00	90	13.20	87
417-87	0.18	100	0.06	100
591-89	20.00	80	9.80	91
4953-88	2.92	101	35.90	103
5538-88	3.73	94	40.96	91
8984-88	29.00	83	8.50	85
Triplet				
2219	25.50	93	9.60	96
2221	22.00	96	12.70	106
2222	23.00	96	8.50	80
Paired strains				
6989	31.00	103	6.06	86
3225	30.00	86	5.30	41

UD - undetectable Sp A - Protein A

Table 31. The effect of 1/2 MIC levels of chloramphenicol on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	50.00	63	6.00	54
E3T	0.97	37	0.19	21
Oxford	0.09	24	UD	_
12009	2.33	76	0.39	37
Wood 46	UD	_	UD	_
Resistant bacteria				
cip 63	20.00	35	8.60	41
cip 86	5.50	88	2.04	55
cip 92	UD	_	UD	_
cip 103	UD	_	UD	_
cip 132	1.95	57	1.60	71
411-87	3.40	35	0.77	61
417-87	0.17	89	UD	_
591-89	5.80	39	2.30	50
4953-88	5.70	68	51.00	85
5538-88	0.36	92	39.00	98
8984-88	15.00	26	9.00	60
Triplet				
2219	15.00	75	3.38	50
2221	14.00	74	2.78	59
2222	18.00	86	5.70	87
Paired strains				
6989	1.30	27	0.60	50
3225	1.70	17	0.60	25

UD - undetectable Sp A - Protein A

Table 33. The effect of 1/4 MIC levels of tetracycline on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	51.00	85	7.20	92
E3T	0.74	102	0.15	38
Oxford	0.36	77	0.11	38
12009	1.18	87	0.46	60
Wood 46	0.34	75	UD	-
Resistant bacteria				
cip 63	25.40	58	4.80	41
cip 86	1.25	82	0.70	99
cip 92	UD	-	UD	-
cip 103	UD	-	UD	-
cip 132	2.90	83	1.06	97
411-87	28.00	93	13.60	90
417-87	0.18	100	UD	-
591-89	15.00	60	5.30	49
4953-88	2.49	86	35.20	101
5538-88	4.00	101	38.40	86
8984-88	22.50	64	7.50	74
Triplet				
2219	26.00	96	8.00	80
2221	22.00	96	8.96	75
2222	17.00	71	5.65	52
Paired strains				
6989	30.00	100	4.50	77
3225	28.00	80	4.30	33

UD - undetectable Sp A - Protein A

Table 33. The effect of 1/2 MIC levels of tetracycline on the production of bound and extracellular protein A

Strain	Bound protein A		Extracellular protein A	
	ng protein A/ mg total cell protein	% of Sp A produced absence	ng protein A/ mg total cell protein	% of Sp A produced absence
Sensitive bacteria				
Cowan 1	45.00	57	6.00	54
E3T	0.85	30	0.09	10
Oxford	0.11	30	UD	_
12009	2.04	67	0.54	51
Wood 46	0.07	27	UD	_
Resistant bacteria				
cip 63	15.00	25	2.50	12
cip 86	1.70	27	0.92	25
cip 92	UD	_	UD	_
cip 103	UD	_	UD	_
cip 132	1.47	43	2.10	90
411-87	3.70	38	0.86	68
417-87	UD	_	UD	_
591-89	2.20	14	1.10	24
4953-88	6.20	73	53.00	88
5538-88	0.42	107	39.00	97
8984-88	6.90	15	7.00	47
Triplet				
2219	13.00	65	4.10	61
2221	13.00	63	2.60	56
2222	14.00	67	2.80	44
Paired strains				
6989	1.30	27	0.20	17
3225	0.40	4	UD	_

UD - undetectable Sp A - Protein A

opposite was seen. Extracellular protein A produced in logarithmic phase, is a secreted form of bound protein A and not a precursor or breakdown product. It would be expected that if production of protein A was reduced, bound protein A would be affected first and then extracellular protein A would be affected. However since extracellular protein A was affected more, it could have been because the levels of extracellular protein A are already low and so any reduction would be more marked. The strains which showed a greater reduction in bound protein A than extracellular protein A, were the strains which produced predominately extracellular protein A. Some strains produce predominately extracellular protein A and these strains are usually MRSA (Winbald and Ericson 1973). Again, the greater effect on cell bound protein A could have been because this was already secreted at low levels and so any reduction would be more marked.

4.3 Production of α haemolysin in the presence of sub-MIC levels of antibiotics

The effect of sub - MIC levels of antibiotics on the production of α haemolysin was investigated. The strains were grown overnight and then subcultured into fresh broth. The strains were then grown for three hours before the antibiotics were added and growth continued for a further two hours. The strains were harvested and measured for α

haemolysin production, the number of the viable cells and total cell protein. The amount of α haemolysin produced in the presence of the antibiotics was compared to the amount produced in the absence of antibiotics. Since α haemolysin is an extracellular enzyme, the level of α haemolysin produced by the strains when related to viable cells will be discussed.

All six antibiotics had some effect on α haemolysin production, both at 1/4 and 1/2 MIC levels, when α haemolysin production was related to viable cells and total cell protein. When α haemolysin production was related to viable cells, the two quinolones, ciprofloxacin and enoxacin, reduced and in some cases completely inhibited α haemolysin production (Tables 34 and 35). At 1/4 MIC levels the effect of these two antibiotics was less than at 1/2 MIC levels. Even when α haemolysin production was related to total cell protein ciprofloxacin and enoxacin still reduced α haemolysin production (appendix Table X). Gentamicin reduced α haemolysin production at 1/2 MIC levels but did not have much effect at 1/4 MIC levels, when α haemolysin production was related to viable cells (Table 36). When α haemolysin production was related to total cell protein, gentamicin still reduced its production but to a lesser extent (appendix Table XI). The other two protein synthesis inhibitors, chloramphenicol and tetracycline, also reduced α haemolysin production and in some cases completely inhibited it (Table 37 and 38).

Table 34. The effect of sub - MIC levels of ciprofloxacin on the production of α haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
Wood 46	1400	67	734	45
E3T	26	34	UD	–
Oxford	29	52	27	25
12009	1100	85	UD	–
Cowan 1	29	60	27	49
Resistant bacteria				
cip 63	50	85	34	56
cip 86	138	66	171	50
cip 92	566	90	278	85
cip 103	24	60	9	17
cip 132	60	87	48	50
411-87	8	14	UD	–
417-87	15	115	15	71
591-89	16	36	UD	–
4953-88	24	63	7	32
5538-88	8	53	UD	–
8984-88	18	25	UD	–
Triplet				
2219	UD	–	UD	–
2221	UD	–	UD	–
2222	UD	–	UD	–
Paired strains				
6989	23	92	39	49
3225	14	82	15	30

UD - undetectable

Table 35. The effect of sub - MIC levels of enoxacin on the production of α haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
Wood 46	1200	57	UD	–
E3T	45	58	UD	–
Oxford	44	78	38	35
12009	540	41	250	26
Cowan 1	46	96	33	61
Resistant bacteria				
cip 63	28	47	UD	–
cip 86	71	34	UD	–
cip 92	627	99	284	87
cip 103	27	67	11	20
cip 132	53	77	27	29
411-87	15	27	UD	–
417-87	6	46	UD	–
591-89	41	93	UD	–
4953-88	23	61	UD	–
5538-88	UD	–	UD	–
8984-88	75	100	UD	–
Triplet				
2219	UD	–	UD	–
2221	UD	–	UD	–
2222	UD	–	UD	–
Paired strains				
6989	12	48	25	31
3225	14	82	21	41

UD - undetectable

Table 36 . The effect of sub- MIC levels of gentamicin on the production of α haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
Wood 46	1800	86	1200	75
E3T	70	91	30	86
Oxford	57	101	72	65
12009	1200	92	900	95
Cowan 1	34	71	35	47
Resistant bacteria				
cip 63	53	90	51	84
cip 86	191	91	257	74
cip 92	500	79	211	65
cip 103	34	85	38	70
cip 132	56	81	46	49
411-87	44	78	58	53
417-87	15	115	19	88
591-89	38	86	31	55
4953-88	41	108	11	50
5538-88	13	87	7	75
8984-88	52	72	36	67
Triplet				
2219	UD	–	UD	–
2221	UD	–	UD	–
2222	UD	–	UD	–
Paired strains				
6989	22	88	60	75
3225	16	94	41	78

UD - undetectable

Table 37. The effect of sub - MIC levels of chloramphenicol on the production of α haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
Wood 46	140	7	UD	–
E3T	57	74	UD	–
Oxford	51	91	32	29
12009	580	45	350	37
Cowan 1	UD	–	UD	–
Resistant bacteria				
cip 63	34	58	23	27
cip 86	110	52	UD	–
cip 92	612	98	44	14
cip 103	28	70	UD	–
cip 132	28	40	28	30
411-87	UD	–	UD	–
417-87	13	100	14	66
591-89	18	41	UD	–
4953 -88	39	102	UD	–
5538-88	UD	–	UD	–
8984-88	26	36	UD	–
Triplet				
2219	UD	–	UD	–
2221	UD	–	UD	–
2222	UD	–	UD	–
Paired strains				
6989	12	48	14	17
3225	14	82	37	72

UD - undetectable

Table 38. The effect of sub - MIC levels of tetracycline on the production of α haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
Wood 46	470	22	UD	–
E3T	17	23	6	18
Oxford	36	64	40	36
12009	820	63	450	47
Cowan 1	UD	–	UD	–
Resistant bacteria				
cip 63	18	31	UD	–
cip 86	31	15	UD	–
cip 92	270	43	UD	–
cip 103	6	15	UD	–
cip 132	30	43	29	32
411-87	53	95	90	83
417-87	UD	–	UD	–
591-89	36	82	UD	–
4953-88	UD	–	UD	–
5538-88	UD	–	UD	–
8984-88	11	15	UD	–
Triplet				
2219	UD	–	UD	–
2221	UD	–	UD	–
2222	UD	–	UD	–
Paired strains				
6989	22	88	13	16
3225	11	65	18	22

UD - undetectable

Table 39. The effect of sub - MIC levels of methicillin on the production of α haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
Wood 46	4300	>200	4248	>200
E3T	2800	>200	252	>200
Oxford	290	>200	460	>200
12009	1600	123	2146	>200
Cowan 1	220	>200	145	>200
Resistant bacteria				
cip 63	100	169	752	>200
cip 86	751	>200	1280	>200
cip 92	1500	>200	2062	>200
cip 103	1100	>200	313	>200
cip 132	65	94	3380	>200
411-87	1400	>200	2230	>200
417-87	4500	>200	4144	>200
591-89	5800	>200	336	>200
4953-88	4000	>200	444	>200
5538-88	310	>200	532	>200
8984-88	68	94	660	>200
Triplet				
2219	UD	–	UD	–
2221	UD	–	UD	–
2222	UD	–	UD	–
Paired strains				
6989	132	>200	330	>200
3225	53	>200	180	>200

UD - undetectable

At 1/2 MIC levels of these two antibiotics, a majority of the strains produced undetectable levels of α haemolysin, if they were producing any α haemolysin at all. At 1/4 MIC levels chloramphenicol and tetracycline still reduced α haemolysin production in most of the strains. Again, when α haemolysin production in the presence of these two antibiotics was related to total cell protein, there was still a reduction in the levels of α haemolysin produced (appendix Table XII). Therefore the reduction in α haemolysin did not result entirely from of a reduction in general protein synthesis. Methicillin greatly increased α haemolysin production by all the strains both at 1/2 and 1/4 MIC levels (Table 39). This antibiotic affects the cell wall and so must be releasing the haemolysin either by lysing the cells or by attacking the wall in some way.

4.4 Production of δ haemolysin in the presence of sub - MIC levels of antibiotics

The production of δ haemolysin was measured in the presence of antibiotics like the other pathogenicity factors studied. Bacterial strains were grown overnight and subcultured into fresh broth. The strains were grown for four hours before the antibiotics were added and growth continued for a further two hours. Strains were also grown in the absence of antibiotics for comparison. The strains were harvested and the production of δ haemolysin, viable cells and total cell protein measured.

The amount of δ haemolysin produced in the presence of the antibiotics was compared to the amount produced in the absence.

The effect of the six antibiotics on δ haemolysin production was similar to the effects seen with α haemolysin. Ciprofloxacin and enoxacin reduced and in some cases completely inhibited δ haemolysin production, when δ haemolysin production was related to viable cells (Tables 40 and 41). Gentamicin had little effect on δ haemolysin production either at 1/2 or 1/4 MIC levels (Table 42). Chloramphenicol and tetracycline again reduced δ haemolysin production, similar to the effects seen with α haemolysin (Tables 43 and 44). Most strains either produced undetectable amounts or no δ haemolysin in the presence of 1/2 MIC levels of these two antibiotics and at 1/4 MIC levels most strains produced significantly reduced amounts of δ haemolysin. Methicillin again had a dramatic effect on the production of δ haemolysin by increasing the amounts of δ haemolysin produced in the presence of 1/2 and 1/4 MIC levels of this antibiotic (Table 45).

4.5 Growth of strains and selection of resistant bacteria in the presence of 1/2-MIC levels of ciprofloxacin

A ciprofloxacin sensitive and a ciprofloxacin resistant strain were grown in the presence of 1/2 MIC levels of ciprofloxacin for 24 hours. Growth was monitored for the first 8 hours by viable counts, taken every hour.

Table 40. The effect of sub - MIC levels of ciprofloxacin on the production of δ haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
E5662	171.0	95	120.0	80
E3T	90.0	81	60.0	65
Oxford	5.2	82	4.8	76
12009	75.0	88	40.0	77
Cowan 1	1.3	74	0.5	50
Wood 46	18.0	90	8.6	61
Resistant bacteria				
cip 63	40.0	35	UD	_
cip 86	57.0	90	35.0	50
cip 92	45.0	88	40.0	38
cip 103	0.8	80	1.0	66
cip 132	52.0	82	26.0	47
411-87	0.8	75	5.5	55
417-87	14.3	70	13.0	61
591-89	UD	_	UD	_
4953-88	UD	_	UD	_
5538-88	0.2	31	UD	_
8984-88	1.1	92	30.0	50
Triplet				
2219	UD	_	UD	_
2221	UD	_	UD	_
2222	UD	_	UD	_
Paired strains				
6989	40.0	83	41.0	73
3225	20.0	69	UD	_

UD - undetectable

Table 41. The effect of sub-MIC levels of enoxacin on the production of δ haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
E5662	110.0	61	110.0	73
E3T	67.0	61	50.0	54
Oxford	2.4	38	UD	–
12009	37.0	43	20.0	38
Cowan 1	0.8	45	UD	–
Wood 46	20.0	100	12.0	85
Resistant bacteria				
cip 63	49.0	43	UD	–
cip 86	58.0	90	40.0	57
cip 92	44.0	69	33.0	31
cip 103	106.0	102	0.8	51
cip 132	58.0	92	20.0	36
411-87	1.0	91	74.0	74
417-87	16.0	79	14.0	66
591-89	UD	–	UD	–
4953-88	UD	–	UD	–
5538-88	UD	–	UD	–
8984-88	1.0	83	40.0	67
Triplet				
2219	UD	–	UD	–
2221	UD	–	UD	–
2222	UD	–	UD	–
Paired strains				
6989	23.0	49	UD	–
3225	26.6	92	25.0	54

UD - undetectable

Table 42. The effect of sub - MIC levels of gentamicin on the production of δ haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
E5662	170.0	95	145.0	97
E3T	105.0	95	87.0	95
Oxford	6.0	94	5.2	83
12009	84.0	99	50.0	96
Cowan 1	1.7	94	1.6	88
Wood 46	18.0	90	12.0	86
Resistant bacteria				
cip 63	110.0	96	90.0	90
cip 86	65.0	101	60.0	86
cip 92	50.0	98	90.0	86
cip 103	1.0	100	1.5	97
cip 132	61.0	97	50.0	91
411-87	1.0	91	3.5	35
417-87	20.4	98	20.0	94
591-89	UD	–	UD	–
4953-88	UD	–	UD	–
5538-88	UD	–	0.5	100
8984-88	1.2	100	5.7	95
Triplet				
2219	UD	–	UD	–
2221	UD	–	UD	–
2222	UD	–	UD	–
Paired strains				
6989	45.0	94	50.0	89
3225	27.0	93	42.0	91

UD - undetectable

Table 43. The effect of sub - MIC levels of chloramphenicol on the production of δ haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
E5662	53.0	30	UD	_
E3T	117.0	106	90.0	98
Oxford	2.8	45	1.1	18
12009	60.0	71	10.0	20
Cowan 1	0.5	28	UD	_
Wood 46	8.0	40	4.0	29
Resistant bacteria				
cip 63	5.5	5	UD	_
cip 86	36.0	56	17.0	24
cip 92	17.0	33	UD	_
cip 103	1.0	97	1.0	63
cip 132	57.0	91	45.0	82
411-87	0.4	40	UD	_
417-87	16.0	78	15.0	70
591-89	UD	_	UD	_
4953-88	UD	_	UD	_
5538-88	0.4	65	0.2	30
8984-88	0.2	19	UD	_
Triplet				
2219	UD	_	UD	_
2221	UD	_	UD	_
2222	UD	_	UD	_
Paired strains				
6989	13.0	27	UD	_
3225	7.0	24	UD	_

UD - undetectable

Table 44. The effect of sub-MIC levels of tetracycline on the production of δ haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
E5662	62.0	35	UD	–
E3T	27.0	25	UD	–
Oxford	3.3	52	1.5	24
12009	58.0	69	UD	–
Cowan 1	1.0	55	UD	–
Wood 46	10.0	50	UD	–
Resistant bacteria				
cip 63	15.1	13	UD	–
cip 86	40.0	63	19.0	27
cip 92	17.0	33	UD	–
cip 103	0.4	38	UD	–
cip 132	10.4	17	UD	–
411-87	0.4	38	UD	–
417-87	15.0	70	UD	–
591-89	UD	–	UD	–
4953-88	UD	–	UD	–
5538-88	UD	–	UD	–
8984-88	0.87	72	4.0	67
Triplet				
2219	UD	–	UD	–
2221	UD	–	UD	–
2222	UD	–	UD	–
Paired strains				
6989	9.5	20	UD	–
3225	5.0	17	UD	–

UD - undetectable

Table 45. The effect of sub - MIC levels of methicillin on the production of δ haemolysin

Strain	1/4 MIC		1/2 MIC	
	HU/10^9 cells	% amount produced in absence	HU/10^9 cells	% amount produced in absence
Sensitive bacteria				
E5662	672	>200	800	>200
E3T	757	>200	205	>200
Oxford	7	113	50	>200
12009	240	>200	380	>200
Cowan 1	3	>200	3	>200
Wood 46	200	>200	280	>200
Resistant bacteria				
cip 63	140	123	200	>200
cip 86	91	140	200	>200
cip 92	112	>200	316	>200
cip 103	7	>200	54	>200
cip 132	500	>200	197	>200
411-87	2	>200	23	>200
417-87	285	>200	95	>200
591-89	2	>200	3	>200
4953-88	UD	_	UD	_
5538-88	2	>200	2	>200
8984-88	0.6	44	57	>200
Triplet				
2219	UD	_	UD	_
2221	UD	_	UD	_
2222	UD	_	UD	_
Paired strains				
6989	304	>200	202	>200
3225	213	>200	196	>200

UD - undetectable

The presence of ciprofloxacin did not seem to affect bacterial growth and the strains did not exhibit slower growth (Figures 25 and 26).

After 24 hours samples were taken from the cultures and plated out on nutrient agar plates containing or lacking ciprofloxacin equivalent to 1x MIC, 2x MIC and 5x MIC. The strains which had been grown in the presence of ciprofloxacin yielded ciprofloxacin resistant mutants on the 1x MIC, 2x MIC and the 5x MIC plates (Table 46). Strains which had not been grown in the presence of ciprofloxacin did not have any resistant mutants. This confirmed that the mutants had been selected by the growth experiment and not by overnight growth on ciprofloxacin containing plates. The resistant mutants were sub-cultured in ciprofloxacin lacking broth for 10 passages to check that they were stable and did not revert back to ciprofloxacin sensitivity.

Table 46. Isolation of resistant mutants after growth for 24 hours in presence of 1/2 MIC levels of ciprofloxacin

Strain	cfu/ml after 24 h	Number of mutants isolated on cipro plates		
		1 x MIC	2 x MIC	5 x MIC
E3T	3.6×10^{10}	51	22	4
cip 103	2.5×10^{10}	21	9	_

Figure 25. Growth of ciprofloxacin sensitive strain in absence and presence of 1/2 MIC ciprofloxacin

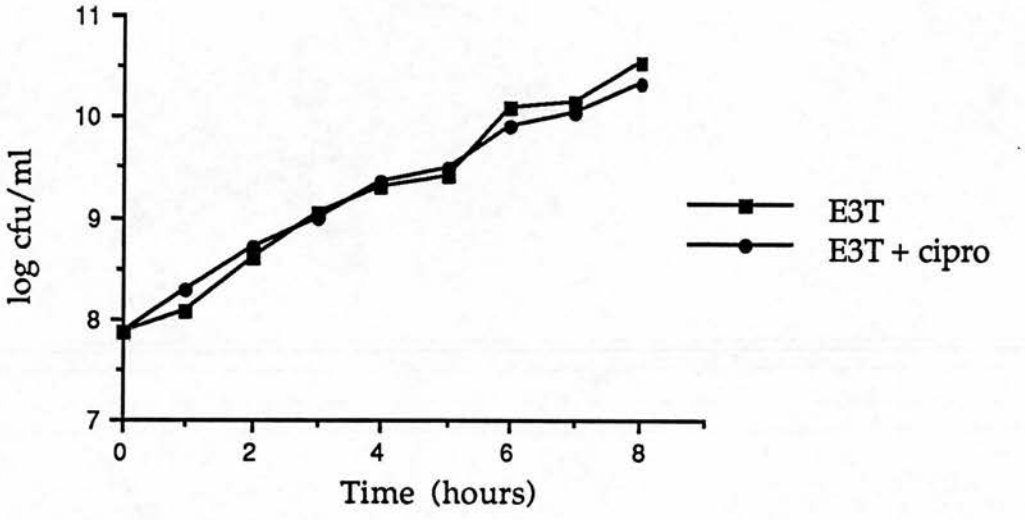
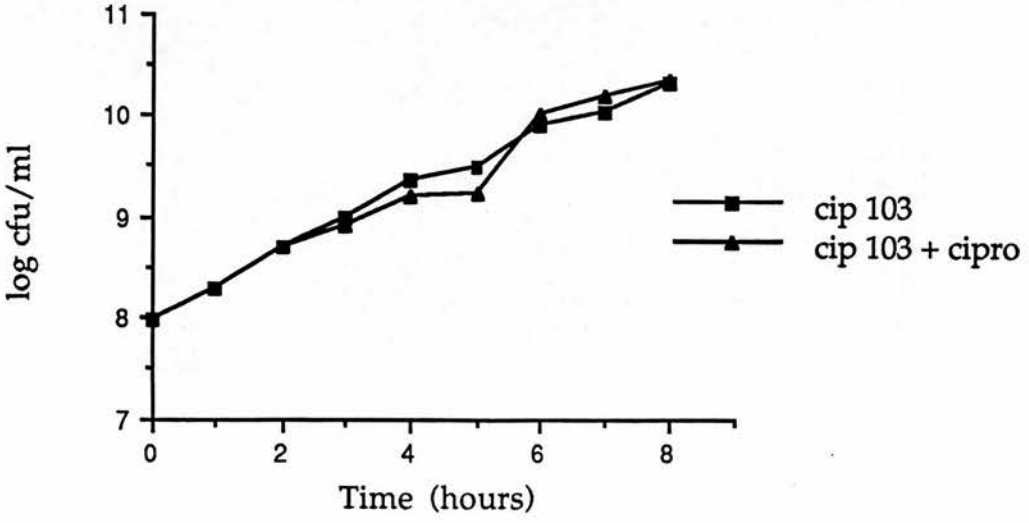


Figure 26. Growth of ciprofloxacin resistant strain in absence and presence of 1/2 MIC ciprofloxacin



4.6 The effect of sub - MIC levels of ciprofloxacin on *in vivo* growth of *S. aureus*

The effect of sub - MIC levels of ciprofloxacin on the development of *S. aureus* in mice was investigated. Before the experiment could proceed, it was necessary to establish the levels of ciprofloxacin which when injected into the mice produced sub-MIC levels of ciprofloxacin in the blood and abscesses. Mice were injected with varying amounts of ciprofloxacin, both intravenously into a vein in the tail and subcutaneously on the left flank. The mice had previously been injected with *S. aureus* on the right flank so that subcutaneous abscesses had developed. Thirty minutes after the ciprofloxacin injections, blood samples were taken ocularly. Three hours after the injections the mice were sacrificed and the abscesses removed. The blood samples and abscess material were analysed by HPLC for level of ciprofloxacin. The levels of ciprofloxacin in the blood and abscesses could be related to the initial injections (Table 47). Three mice were sacrificed for each ciprofloxacin concentration.

By employing the figures of ciprofloxacin levels in the serum and abscesses as guidelines, the amount of ciprofloxacin needed to achieve 1/2 MIC levels of the drug in the abscesses was calculated.

Mice were injected with *S. aureus* as described in section 2.4.15 and, one

Table 47. Levels of ciprofloxacin (mg/L) in the blood and abscesses after injection by different routes

Initial injection of ciprofloxacin (mg/kg body weight)	Level of ciprofloxacin (mg/L) intravenous injection		sub-cutaneous injection	
	Blood	Abscess	Blood	Abscess
20	2.4	2.5	4.5	4.7
12	1.2	1.5	1.9	2.3
8	0.7	0.7	1.0	1.5
4	0.4	0.6	0.6	0.9
2	0.2	0.3	0.3	0.5

hour after this subcutaneous injection, mice were injected with 1/2 MIC levels of ciprofloxacin. Since ciprofloxacin has a half life of 60 minutes in serum of mice (information obtained from Bayer UK) the mice were injected with ciprofloxacin at two hourly intervals, so that the level of ciprofloxacin remained near 1/2 MIC levels. These levels were maintained for 8 hours and then resumed 16 hours later, since injections were not given during the night. The mice were given two injections the following day, to maintain the selective pressure, before being sacrificed. The abscesses were excised and viable counts obtained from them as described in section 2.4.16. Treatment with ciprofloxacin at sub-MIC levels reduced the number of organisms recovered from the abscesses (student t test $p < 0.01$), but 1/4 MIC levels did not reduced the number of bacteria recovered greatly (Table 48).

Table 48. Treatment of mice with sub MIC levels of ciprofloxacin for 24 hours

Number of viable bacteria after 24 hours (cfu/ml)			
Control (no drug)	1/2 MIC	Control (no drug)	1/4 MIC
5.0×10^7	1.0×10^6	5.0×10^7	3.5×10^7
6.5×10^7	1.0×10^7	2.5×10^8	7.0×10^6
1.8×10^8	4.0×10^6	3.5×10^7	2.8×10^7
1.4×10^8	5.0×10^5	2.0×10^7	3.1×10^7
7.5×10^7	6.0×10^6	7.0×10^7	1.6×10^7
2.7×10^7	2.0×10^6	3.9×10^7	7.0×10^6
7.5×10^7	2.0×10^6	4.0×10^7	3.0×10^7
2.2×10^7	1.0×10^6	6.0×10^7	8.0×10^6
5.0×10^7	7.0×10^6	8.0×10^7	2.8×10^7
7.0×10^7	3.0×10^6	2.8×10^7	4.0×10^7
Mean			
7.5×10^7	3.6×10^6	6.7×10^7	4.0×10^7

In a subsequent experiment, mice were injected with *S. aureus* as described in section 2.4.15 and the abscesses were allowed to develop for 48 hours. After this time, treatment with 1/2 MIC levels of ciprofloxacin for the strain injected (strain 6989 - ciprofloxacin sensitive) was started. The treatment was continued for 24 hours as described in the previous paragraph, with two hourly injections on the left flank. After the 24 hours of treatment was completed, the mice were sacrificed, the abscesses removed and viable counts obtained from them as described in section 2.4.16. The abscess material was also plated out on agar plates containing ciprofloxacin at 2 x MIC and 4 x MIC for strain 6989, to enable the selection of ciprofloxacin resistant mutants which had developed during the treatment. Abscess material from mice not treated with ciprofloxacin was also plated out, to ensure that the resistant mutants had not been selected out by overnight growth on the ciprofloxacin plates. Treatment with ciprofloxacin led to the selection of isolates resistant to 2 x MIC and 4 x MIC (Table 49).

4.7 Summary

The effects of sub-MIC levels of six antibiotics on the pathogenicity of *S. aureus* was investigated. The six antibiotics used were ciprofloxacin, enoxacin, gentamicin, chloramphenicol, tetracycline and methicillin.

Coagulase production was affected by 1/4 and 1/2 MIC levels of

Table 49. Selection of ciprofloxacin resistant bacteria after 24 hours treatment with ciprofloxacin

Untreated mice		Treated mice	
Viable bacteria	cipro resistant	Viable bacteria	cipro resistant
6.4×10^7	_	9.1×10^7	19
7.0×10^7	_	5.1×10^7	_
1.0×10^8	_	9.5×10^7	11
2.0×10^8	_	5.4×10^7	4
3.5×10^7	_	1.7×10^8	15
5.0×10^7	_	8.3×10^7	12
7.9×10^7	_	7.0×10^7	_
6.5×10^7	_	9.3×10^7	14
6.2×10^7	-	1.5×10^8	15
8.1×10^7	_	3.5×10^8	17

gentamicin, chloramphenicol and tetracycline. Ciprofloxacin, enoxacin and methicillin had little or no effect on coagulase production. Protein A production was not affected by 1/4 and 1/2 MIC levels of ciprofloxacin, enoxacin and methicillin. However, chloramphenicol and tetracycline reduced the production of both bound and extracellular protein A at 1/4 and 1/2 MIC levels. Gentamicin also reduced protein A production but not to the same extent as the other two protein synthesis inhibitors.

The two haemolysins of all strains were affected by the six antibiotics in a similar manner. Ciprofloxacin, enoxacin, chloramphenicol and tetracycline at 1/2 MIC levels reduced and in some cases completely inhibited the production of the two haemolysins. At 1/4 MIC levels of these four antibiotics α and δ haemolysin production was reduced to a lesser extent. Gentamicin had little or no effect on α or δ haemolysin production. Methicillin at 1/2 and 1/4 MIC levels greatly increased α or δ haemolysin production.

The effect of sub-MIC levels of ciprofloxacin on the growth of S. *aureus* both *in vitro* and *in vivo* was investigated. *In vitro*, 1/2 MIC levels of ciprofloxacin had little or no effect on the growth over 8 hours. After 24 hours, ciprofloxacin resistant mutants of the strains were isolated from the culture medium. *In vivo*, treatment with 1/2 MIC levels of ciprofloxacin reduced the number of bacteria recovered from the

abscesses when treatment was started one hour after the sub-cutaneous injection of the bacteria. Ciprofloxacin at 1/4 MIC levels had little or no effect on the abscesses.

5 Discussion

Microbial pathogenicity is complex and multifactorial (Smith 1958). Pathogens often have several biochemical mechanisms which may act individually or together to produce disease. Because of the multifactorial nature of pathogenic mechanisms, they have been studied in many ways. The organisms can be altered, so that they lack one particular feature - toxin, adhesin etc, and then this organism can be examined *in vitro* and *in vivo* (Jonsson *et al* 1985; Patel *et al* 1987; Bramley *et al* 1989). Virulent or avirulent strains can be compared for their production of particular toxins etc. Another method is to investigate the effect of antibiotics on virulence factor production (Lorian 1986). Antibiotics are used at concentrations below those required to inhibit growth (subminimum inhibitory concentrations - sub - MIC), since at concentrations higher than this, i.e. equal to the MIC, the antibiotic completely inhibits the growth of bacteria and therefore eliminates their virulence. Since it has been realised that human infections increasingly result from antibiotic resistant organisms, work has concentrated on the pathogenicity of these resistant organisms, as a knowledge of their products and role in pathogenesis may produce information of direct medical importance.

The effect of antibacterials on the pathogenicity of *S. aureus* was examined in this project. The effect of both sub-inhibitory concentrations

of certain antibiotics on *S. aureus* was studied, as well as the effect of antibiotic resistance on pathogenicity. It has been reported that strains which have acquired antibiotic resistance, either plasmid-mediated or by chromosomal mutations, exhibit reduced virulence (Rake *et al* 1944; Blair *et al* 1946; Lacey and Chopra 1975; Musher *et al* 1977). In the light of some preliminary reports concerning various bacterial species, it was speculated that *S. aureus*, which had developed chromosomal resistance to the 4-quinolone group of antibacterials, might also exhibit reduced virulence (Ravizzola *et al* 1987; Crumplin 1987; Smith 1990). These resistant bacteria were investigated, both *in vitro* and *in vivo*, to see if they were more or less pathogenic than sensitive bacteria. They were also compared to high factor producing strains. The pathogenicity of the strains in the presence of sub-minimum inhibitory concentrations (sub-MICs) of the 4-quinolones and other antibiotics was also examined, since resistant strains sometimes are less virulent in the presence of antibiotics (Nordstrom and Lindberg 1978).

5.1 Pathogenicity of ciprofloxacin resistant *S. aureus*

Ciprofloxacin-resistant clinical isolates of *S. aureus* were compared to sensitive strains for their pathogenicity. The growth rates of the strains were analysed along with the production of four virulence factors. The four factors studied in detail were coagulase, protein A, α haemolysin and δ haemolysin. These four factors were chosen since they are generally produced by most *S. aureus* strains and also for their assorted

effects on the host defence system. No one virulence factor can be solely attributed to the pathogenicity of *S. aureus*, since the pathogenicity of *S. aureus* is like pathogenicity in general - multifactorial and a result of many components.

5.1.1 Growth of ciprofloxacin sensitive and resistant strains

The growth of the resistant and sensitive strains was studied, since growth rate is important in pathogenicity (Smith 1990). A slow growing pathogen will be overcome by the host defence mechanisms before it has had a chance to cause disease. Avirulence can arise from inability of bacteria to grow and divide (Smith 1968). Also, differences in the production of toxins and enzymes might be a result of a slow growth rate, rather than actual differences in toxin production.

The resistant and sensitive strains grew at similar rates during logarithmic phase, which supports the views of Kayser and Novak (1987). They also grew ciprofloxacin sensitive and resistant strains of *S. aureus* and found no apparent differences.

Ban (1981) observed that in a mixed culture of penicillin resistant and sensitive strains, the resistant strains grew at a higher specific growth rate and that the ratio of resistant to sensitive bacteria changed from 1:1 to 1:1.5 over the 24 hour period. This was in contrast to Lacey and Chopra (1975) who found that in a mixed culture of resistant and sensitive

bacteria, the sensitive strains grew at a faster rate over 24 hours. In the current study, when growth was continued in stationary phase, still no differences were seen; indeed when replication rates of a mixture of ciprofloxacin sensitive and resistant strains were studied, the ciprofloxacin resistant strain persisted in the mixture, confirming that it had a similar growth rate.

Nalidixic acid resistant mutants of *E. coli* and *Salmonella typhimurium* lost the ability to grow anaerobically and so become obligate aerobes (Yamamoto and Droffner 1985; Yoshizawa and Yamamoto 1989). Some of the ciprofloxacin resistant strains were grown anaerobically, to establish if they too had lost the ability to grow under anaerobic conditions. However, they retained the ability to grow in the absence of oxygen.

These quinolone resistant strains grew at similar rates to the sensitive strains both in the logarithmic and stationary phases. They persisted in a mixed culture of sensitive and resistant strains and they retained the ability to grow under anaerobic conditions.

5.1.2 Production of virulence factors by sensitive and resistant strains

It has been observed previously that antibiotic resistant bacteria are less pathogenic than their sensitive counterparts. Gentamicin resistant *S. aureus* grew as small nonhaemolytic colonies, producing low levels

of DNase, mannitol and coagulase (Musher *et al* 1977). These gentamicin resistant strains were shown to be less virulent in rats and mice (Musher *et al* 1977; Pelletier *et al* 1979). Penicillin resistant *S. aureus* produced lower levels of coagulase and protein A (Rake *et al* 1944; Blair *et al* 1946). A similar situation was observed in methicillin resistant *S. aureus* (Barber 1961; Knox and Smith 1961). Jordens *et al* (1989) found that epidemic MRSA produced higher levels of coagulase, but similar levels of the haemolysins. Roberts and Gaston (1987) also found that higher levels of coagulase were produced by epidemic MRSA and that these strains produced low levels of protein A. Mutants of *S. aureus* resistant to ofloxacin were reported not to produce coagulase (Smith 1990), but this was a study of a small number of laboratory mutants and they were tested by the slide coagulase test. The slide coagulase test detects bound coagulase production only and often leds to false negative results. Lacey and Chopra (1975) suggested that acquisition of resistance plasmids by *S. aureus* is associated with a decrease in virulence. A similar study by Cutler (1979) could not corroborate this; indeed, he showed the opposite, that the loss of bacterial virulence was associated with the loss of antibiotic resistance determinants. Ishag and Shibl (1985) also found no correlation between patterns of resistance and the production of certain toxins and enzymes by clinical isolates of *S. aureus*.

The production of the four virulence factors studied was measured

during optimum production of the factor concerned. Bound and soluble coagulase production was measured employing a much improved chromogenic assay for coagulase. Bound and extracellular protein A production was measured, since extracellular protein A production can be as high as 30% of total protein A output in some strains and is much enhanced in some MRSA strains (Winbald and Ericson 1973). Only the extracellular production of the two haemolysins was studied since they are considered as extracellular enzymes.

The 4-quinolone resistant strains tested in this study produced similar levels of the four factors to the sensitive strains. Four strains were acquired which produced high levels of each of the four factors studied. Ciprofloxacin resistant mutants of these strains were made and tested for the production of the factors. No differences were seen, suggesting that in *S. aureus,* ciprofloxacin resistance does not affect pathogenicity. In order to confirm these results, the behaviour of the strains *in vivo* was investigated.

5.1.3 Pathogenicity of resistant and sensitive strains *in vivo*

The necessity to study pathogenicity *in vivo* as well as *in vitro* has been well documented (Smith 1968; Shibl 1983). Bacteria often behave differently *in vivo* and *in vitro,* producing different levels of toxins, surface antigens etc. *S. aureus* grown *in vivo* have different surface antigens (Karakawa and Kane 1975; Watson and Prideaux 1979), produce

different levels of DNase, leucocidin and α haemolysin (Beining and Kennedy 1963).

A selection of the resistant and sensitive strains were compared *in vivo,* in a sub-cutaneous abscess model in mice. This model allowed easy comparison of strains, as they grew *in vivo,* by monitoring the development of the abscesses. The resistant strains produced similar abscesses to the sensitive strains - both in size and degree of tissue damage, suggesting that they were of similar virulence. Lacey and Chopra (1975) found that methicillin resistant *S. aureus* were less virulent for mice. Ciprofloxacin resistant *Ps. aeruginosa* were also found to be less virulent for mice (Ravizzola *et al* 1987). The results from this study suggest that for *S. aureus,* ciprofloxacin resistant strains have similar virulence for mice. They would support findings of Ishag and Shibl (1985) who found no correlation between patterns of resistance and the production of virulence factors.

5.2 Pathogenicity of bacteria in the presence of sub-MIC s of antibiotics

Dosage levels of antimicrobial agents are often determined by the use of parameters which have little bearing on the drug levels found at the site of infection. In fact antibiotics are often present in sub-MIC concentrations at the site of infection (Zak and Kradolfer 1979).

Therefore, it is of interest to investigate the effect of sub-MIC levels of antibiotics on the virulence of microorganisms. If the various components of virulence eg. growth rate, toxin production etc, are altered by these levels of antibiotics *in vitro*, this might also influence the virulence of the organism *in vivo* and alter the host's ability to respond to the infection (Gemmell 1982).

The study was primarily concerned with the effects of sub-MIC levels of the quinolones on virulence. However, to enable comparisons with antibiotics with different modes of action, the effects of some protein synthesis inhibitors and cell wall synthesis inhibitors were also investigated. The antibiotics investigated were ciprofloxacin, enoxacin, gentamicin, chloramphenicol, tetracycline and methicillin.

5.2.1 Coagulase Production

The three protein synthesis inhibitors, gentamicin, chloramphenicol and tetracycline reduced coagulase production at 1/4 and 1/2 MIC levels. The amount of coagulase produced in the presence of the antibiotics was initially related to viable cells but then it was also related to total cell protein. The reduction in coagulase production was still observed when related to total cell protein which suggests that besides affecting protein synthesis, and therefore coagulase production, these antibiotics affect some other aspect of the secretion or synthesis of coagulase. Growth of *S. aureus* in the presence of sub-MIC levels of chloramphenicol and

tetracycline leads to a 3 - 4 fold thickening of the cell wall (Hash and Davis 1962; Cardieuz *et al* 1970). This might reduce or prevent the secretion of coagulase and other extracellular enzymes by the cell. Gemmell and Shibl (1976) also showed that chloramphenicol, at sub-MIC levels, reduced the production of coagulase. Lincomycin and clindamycin were also shown to have a limiting effect on coagulase production.

Several investigators have observed that exoenzyme synthesis is considerably more sensitive to antibiotic inhibition than is general protein synthesis (Stinson and Merrick 1974; Boethling 1975; Shibl 1977). This phenomenon has led to the hypothesis that extracellular products are synthesised or assembled on the surface of cell membranes (Shibl 1983). It has been shown that polyribosomes participating in the synthesis of secretory proteins are tightly bound to the membrane (Randall and Hardy 1977). Membrane-bound ribosomes are selected by mRNA molecules, since they possess a unique sequence of codons, which are not present in mRNAs coding for cytoplasmic proteins (Blobel and Dobberstein 1975). Differential inhibition of the synthesis of *E. coli* envelope proteins as compared with the synthesis of cytoplasmic proteins, was observed in the presence of various ribosome specific antibiotics e.g. chloramphenicol and tetracycline (Hirashima *et al* 1973). Membrane-bound ribosomes are localised peripherally and are thus more sensitive to inhibition by antibiotics than are cytoplasmic

ribosomes. Antibiotics which do not inhibit extracellular products may actually act on bacterial protein synthesis in general and not specifically on proteins whose origins are at or near the cell membrane (Shibl 1983). The effect of chloramphenicol and tetracycline on coagulase production support this idea of membrane bound ribosomes, since coagulase production was reduced more than the general protein synthesis.

5.2.2 Protein A production

Protein A is a cell wall protein which is maximally produced during exponential growth phase (Forsgren 1969). Extracellular protein A has also been detected in culture supernatants and its production follows the pattern of cell bound protein A (Movitz 1976). He showed that extracellular protein A produced during exponential growth is neither a precursor nor a breakdown product of bound protein A - it is a true extracellular protein. In stationary phase, the situation is completely different and high levels of extracellular protein A are produced, as a result of cell lysis and degradation of the cell wall (Movitz 1976). Protein A production was measured during exponential growth and both types of protein A were measured to see if the antibiotics tested had different effects on protein A synthesis.

Streptomycin and novobiocin increased protein A production in resistant mutants, and after two passages in medium containing antibiotic, protein A production was increased even more (Nordstrom

and Lindberg 1978). Gemmell and O'Dowd (1983) investigated protein A production and tried to relate it to the phagocytosis of bacteria by leucocytes. They showed that clindamycin and fusidic acid reduced the production of protein A and increased the susceptibility of *S. aureus* to phagocytosis. It has also been demonstrated that 1/8 MIC levels of the quinolones increased ingestion of *S. aureus* by PMN and speculated that this might be a result of lower levels of protein A produced in the presence of quinolones.

This study shows that the quinolones did not have any effect on the production of protein A and so the increased ingestion of *S. aureus* must be a result of another factor. Again all three protein synthesis inhibitors greatly affected both types of protein A synthesis. Chloramphenicol and tetracycline had a greater effect than gentamicin. The production of extracellular protein A was affected to a greater extent than the production of cell bound protein A. Since extracellular protein A is a secreted form of cell bound protein A, any reduction in the levels of cell bound protein A will affect extracellular protein A production. That extracellular protein A production is affected to a greater degree could be a result of one of two reasons. If extracellular protein A is secreted as a 'surplus' form of bound protein A, when the levels of bound protein A are reduced, extracellular protein A will not be secreted to the same extent, so that normal levels of cell bound protein A will be produced. Also in most strains, low levels of extracellular protein A are produced

and any reduction in these levels will seem more noticeable.

Methicillin had little or no effect on protein A production which is surprising since its mechanism of action is to inhibit cell wall synthesis. It could have been expected that it might interfere with the incorporation of protein A into the cell wall, thus reducing levels of cell bound protein A but increasing levels of extracellular protein A. Movitz (1974) showed that protein A production continued when peptidoglycan synthesis ceased when strains were grown in the presence of vancomycin. The bacteria could still incorporate protein A into the membrane despite no peptidoglycan synthesis. The results from this study suggest that in the presence of methicillin, protein A production continues and is incorporated into the cell wall normally. It would be interesting to observe the effects of longer exposure to methicillin on protein A production, since a time interval of more than 2 hours might have a more pronounced effect on protein A production.

5.2.3 Haemolysin Production

α haemolysin and δ haemolysin will be discussed together since they were affected similarly by the six antibiotics studied. Both these haemolysins are produced mainly in stationary phase, and because of this their production was measured in late exponential and early stationary phase.

Chloramphenicol and tetracycline inhibited haemolysin production whereas gentamicin had little affect. Chloramphenicol and tetracycline have been shown previously to inhibit haemolysin production (Hinton and Orr 1960; Gemmell and Shibl 1976). This reduction was not just a result of decreased protein synthesis because this project has shown that α and δ haemolysin in the presence of chloramphenicol and tetracycline when related to total cell protein is still reduced. Some mechanisms other than reduced protein synthesis must be affecting α and δ haemolysin synthesis. Again considering that *S. aureus* grown in the presence of chloramphenicol and tetracycline have thickened cell walls, this might reduce haemolysin synthesis. The effect of these antibiotics on membrane bound ribosomes, as discussed previously, might also have some effect on haemolysin synthesis. Gentamicin had a reduced effect on haemolysin synthesis - despite also affecting protein synthesis. This could be because gentamicin does not affect the cell wall like the other protein synthesis inhibitors studied and so the reduction in haemolysin production is solely a result of reduced protein synthesis. The relation of haemolysin production to viable cells and total cell protein help support this idea because, when haemolysin production is related to total cell protein, the levels are much nearer 100% of the normal levels than the viable count figures. Again, it might result from gentamicin not affecting membrane bound ribosomes but affecting general protein synthesis instead.

The production of the haemolysins in the presence of methicillin was greatly increased. This observation has been noted previously (Kobayasi *et al* 1966) and is probably a result of methicillin's mechanism of action - affecting cell wall synthesis. Penicillin has a similar effect on haemolysin production (Kobayasi *et al* 1966). By attacking the cell wall it releases the toxins into the surrounding medium, so accelerating the secretion process.

The two quinolones greatly reduced the production of the two haemolysins - to undetectable levels in some strains. The quinolones initiate cell death by binding to DNA gyrase (Wolfson *et al* 1990). The actual mechanism of killing is multifactorial involving the prevention of DNA synthesis, additionally at higher concentrations a reduction of RNA and protein syntheses occurs (Wolfson *et al* 1990). At sub - MIC levels, DNA synthesis might be affected and then in turn RNA synthesis. Therefore the production of the haemolysins would be affected. However, it has been reported that the quinolones have little killing power on stationary phase bacteria, since DNA synthesis is generally reduced during this period (Chalkley and Koornhof 1985).

In *S. aureus*, as in most other bacteria, pathogenicity is multifactorial and the genes that encode the virulence factors are subject to coordinated regulation. These regulatory systems are supposed to respond to changes in the bacteria's surroundings during the infection process. The majority

of extracellular proteins from *S. aureus* are produced preferentially at the end of exponential phase (Abbas-Ali *et al* 1977). The synthesis of at least 14 extracellular toxins and enzymes is regulated by a set of trans-acting elements from the *agr* locus (Recsei *et al* 1986). Coagulase and protein A synthesis are negatively regulated by *agr*, whereas the haemolysins, lipase, TSST-1 etc, positively regulated by it (Kornblum *et al* 1990). The actual mechanism by which this regulatory system is controlled in *S. aureus* is unknown. Various other virulence genes are controlled by regulatory systems eg. toxin genes of *Vibrio cholera* (Peterson and Mekallanos 1988) and the virulence genes of *Bordetella pertussis* (Stibitz *et al* 1989) and they respond to environmental signals such as osmolarity, anaerobiosis and temperature. It has been suggested that DNA supercoiling is involved in this regulation of gene expression (Dorman *et al* 1988; Higgins *et al* 1988). It has previously been suggested that the *S. aureus agr* regulatory system is sensitive to fluctuations in DNA topology (Dorman *et al* 1991). Nordstrom and Lindberg (1978) found an inverse correlation between protein A synthesis (negatively regulated by *agr*) and the production of α and β haemolysin (positively regulated by *agr*) in the presence of novobiocin, an antibiotic which affects DNA supercoiling. The production of protein A was stimulated and the production of the haemolysins was reduced. It is interesting to note that the production of the two virulence factors investigated in this project (coagulase and protein A), which are negatively controlled by *agr* , were not affected by the quinolones over

the time period studied, but the two haemolysins which are positively regulated by *agr*, are affected by the quinolones. Obviously, this could be a result of the quinolones having an effect on factors produced in late exponential phase or early stationary phase and nothing to do with *agr*. To accumulate more evidence, the production of some other factors affected or not affected by *agr* should be investigated in the presence of the quinolones. Enterotoxin A which is neither positively or negatively regulated by *agr* would be a good candidate for this, especially since it is produced in the stationary phase. Also the production of coagulase and protein A in the presence of quinolones over a longer time period than one investigated in this study, should be considered.

5.2.4 Growth of bacteria in the presence of sub-MIC levels of ciprofloxacin

Since the study was mainly concentrating on the effect of ciprofloxacin on pathogenicity, the final section considers the growth of strains in the presence of ciprofloxacin.

When strains were grown in the presence of 1/2 MIC levels of ciprofloxacin, they grew at a similar rate to strains grown in the absence of ciprofloxacin. Slower growth rates would be expected in higher concentrations of ciprofloxacin as observed by Chalkley and Koornhof (1985). At 1/2 MIC levels and for 8 hours only, no real effects on growth

Table 50. The effects of sub-MICs of antibiotics on production of toxins and enzymes by *S. aureus*

Protein	Increase	Decrease	Reference
Coagulase		chloramphenicol	This Study/Gemmell and Shibl (1976)
		gentamicin	This Study
		tetracycline	This Study
		clindamycin	Gemmell and Shibl (1976)
		lincomycin	
Protein A		chloramphenicol	This Study/Gemmell and Shibl (1976)
		tetracycline	This Study
		gentamicin	This Study
		clindamycin	Gemmell and O'Dowd (1983) " "
		fusidic acid	
	streptomycin		Nordstrom and
	novobiocin		Lindberg (1978)
α toxin	methicillin		This Study/Kobayasi *et al* (1966)
		quinolones	This Study
		chloramphenicol	This Study/Gemmell and Shibl (1976);Hinton and Orr (1960)
		tetracycline	This Study
		streptomycin	Nordstrom and Linberg (1978)
		novobiocin	" "
		clindamycin	Gemmell (1983)
		lincomycin	"
δ toxin	methicillin		This Study
		quinolones	This Study
		chloramphenicol	This Study
		tetracycline	This Study

rates would be expected. When 1/2 MIC levels of ciprofloxacin were injected into mice, the resultant abscesses produced less tissue damage and the number of viable bacteria recovered was considerably less. If the growth of the strains *in vivo* was also not affected by 1/2 MIC levels then the reduction in the number of bacteria recovered could have been a result of reduced virulence, reduced production of virulence factors or maybe *in vivo* 1/2 MIC levels of ciprofloxacin do reduce the growth rate.

After growth in 1/2 MIC levels of ciprofloxacin both *in vitro* and *in vivo*, ciprofloxacin resistant strains were isolated. These strains had been selected out by growth in sub-MIC levels of ciprofloxacin. The strains were stable mutations because even after repeated subculture in medium lacking ciprofloxacin, they were still resistant to ciprofloxacin. There have been some reports of treatment failure in patients as a result of ciprofloxacin resistant strains of *S. aureus* developing during therapy (Humphreys and Mulvihill 1985; Milne and Faires 1988). Obviously this is an area of concern and highlights the need for careful control of ciprofloxacin administration, so that sub MIC levels of the drug are not consistently achieved. The work presented here suggests that these quinolone resistant bacteria are just as virulent as the sensitive strains and will persist in the infection site.

5.3 Conclusions

1. Ciprofloxacin resistant strains of *S. aureus* are no less pathogenic than ciprofloxacin sensitive strains -

They grow at similar growth rates both in logarithmic phase and stationary phase. They are still able to grow under anaerobic conditions.

They produce similar amounts of the four virulence factors studied - coagulase, protein A, α haemolysin and δ haemolysin.

When grown *in vivo*, they produced subcutaneous abscesses in mice of similar size and degree of tissue damage.

2. Sub-minimum inhibitory concentrations of the quinolones reduced the production of the haemolysins, whereas the protein synthesis inhibitors, chloramphenicol, tetracycline and gentamicin reduced the production of all the factors studied.

Growth *in vitro* and *in vivo*, in the presence of 1/2 MIC levels of ciprofloxacin, was not affected and resulted in the selection of bacteria stably resistant to ciprofloxacin.

3. Future work could involve investigating more accurately the effect of ciprofloxacin resistance on pathogenicity of *S. aureus*, by making genetically engineered isogenic strains differing solely their sensitivity to ciprofloxacin. Therefore, any differences in the pathogenicity of the strains would be a result of resistance to ciprofloxacin.

The effect of sub-MIC levels of ciprofloxacin on the regulation of *agr* could be investigated further to see if changes in DNA supercoiling affected the regulation of *agr*. Production of enterotoxin A in the presence of sub-MIC levels of ciprofloxacin would give indications of this, since enterotoxin A production is neither positively or negatively regulated by *agr*.

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Appendix

Table I. The effect of 1/4 MIC levels of ciprofloxacin on the production of coagulase when related to viable cells

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% amount produced in absence	ng coagulase/ 10^9 cells	% amount produced in absence
Sensitive bacteria				
12009	23.40	104	7.31	106
E3T	4.74	103	1.41	101
Oxford	6.53	99	3.01	97
Cowan 1	9.09	101	0.96	107
Wood 46	2.60	104	0.92	102
Resistant bacteria				
cip 63	14.66	104	5.15	103
cip 86	5.14	107	1.20	109
cip 92	2.55	98	1.07	107
cip 103	5.56	101	1.55	103
cip 132	4.59	102	0.61	101
411-87	9.90	99	2.85	95
417-87	9.41	99	2.42	101
591-89	2.06	103	1.46	104
4953-88	5.25	105	1.17	106
5538-88	7.17	107	1.43	102
8984-88	3.61	103	0.92	102
Triplet				
2219	11.51	101	3.09	103
2221	11.22	102	4.04	101
2222	10.03	109	4.60	107
Paired strains				
6989	6.45	104	1.61	107
3225	6.90	103	3.57	102

Table II. The effect of 1/4 MIC levels of enoxacin on the production of coagulase when related to viable cells

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% amount produced in absence	ng coagulase/ 10^9 cells	% amount produced in absence
Sensitive bacteria				
12009	22.95	102	6.97	101
E3T	4.51	98	1.36	97
Oxford	6.73	102	3.19	103
Cowan 1	9.36	104	0.92	102
Wood 46	2.68	107	0.98	109
Resistant bacteria				
cip 63	13.96	99	5.05	101
cip 86	4.66	97	1.06	96
cip 92	2.63	101	1.05	105
cip 103	5.89	107	1.59	106
cip 132	4.37	97	0.64	107
411-87	10.10	101	3.06	102
417-87	9.88	104	2.45	102
591-89	2.02	101	1.44	103
4953-88	5.20	104	1.18	107
5538-88	7.30	109	1.54	110
8984-88	3.57	102	0.94	104
Triplet				
2219	11.97	105	3.21	107
2221	11.99	109	4.40	110
2222	9.38	102	4.47	104
Paired strains				
6989	6.63	107	1.53	102
3225	7.10	106	3.82	109

Table III. The effect of 1/4 MIC levels of methicillin on the production of coagulase when related to viable cells

Strain	Soluble coagulase		Bound coagulase	
	ng coagulase/ 10^9 cells	% amount produced in absence	ng coagulase/ 10^9 cells	% amount produced in absence
Sensitive bacteria				
12009	22.28	99	6.76	98
E3T	4.65	101	1.44	103
Oxford	6.86	104	3.32	107
Cowan 1	9.81	109	0.99	110
Wood 46	2.55	102	0.94	104
Resistant bacteria				
cip 63	14.81	105	5.30	106
cip 86	5.14	107	1.21	110
cip 92	2.63	101	1.02	102
cip 103	5.72	104	1.58	105
cip 132	4.77	106	0.66	110
411-87	10.40	104	3.15	105
417-87	9.69	102	2.50	104
591-89	1.82	91	1.39	99
4953-88	5.20	104	1.12	102
5538-88	6.90	103	1.50	107
8984-88	3.57	102	0.94	104
Triplet				
2219	11.74	103	3.21	107
2221	11.88	108	4.28	107
2222	9.84	107	4.47	104
Paired strains				
6989	6.32	102	1.55	103
3225	7.17	107	3.71	106

Table IV. The effect of sub-MIC levels of ciprofloxacin on the production of coagulase when related to total cell protein

Strain	1/2 MIC		1/4 MIC	
	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein
Sensitive bacteria				
12009	3.20 (99)	0.81 (101)	3.42 (106)	0.83 (104)
E3T	2.15 (101)	0.33 (103)	2.17 (102)	0.32 (99)
Oxford	1.69 (105)	0.32 (99)	1.58 (98)	0.32 (99)
Cowan 1	2.17 (98)	0.44 (101)	2.23 (101)	0.45 (103)
Wood 46	0.83 (99)	0.22 (102)	0.83 (97)	0.21 (97)
Resistant bacteria				
cip 63	2.56 (89)	0.88 (102)	2.79 (97)	0.90 (105)
cip 86	1.61 (106)	0.36 (114)	1.54 (101)	0.35 (109)
cip 92	1.47 (105)	0.26 (116)	1.32 (102)	0.22 (98)
cip 103	2.67 (101)	0.63 (96)	2.72 (103)	0.69 (104)
cip 132	2.67 (103)	0.48 (108)	2.72 (105)	0.47 (107)
411-87	2.51 (98)	0.63 (92)	2.53 (99)	0.70 (101)
417-87	2.42 (99)	0.67 (103)	2.54 (103)	0.65 (105)
591-89	1.56 (98)	0.34 (106)	1.70 (107)	0.35 (109)
4953-88	1.99 (97)	0.34 (110)	2.03 (99)	0.30 (98)
5538-88	2.76 (101)	0.51 (104)	2.59 (95)	0.49 (99)
8984-88	1.88 (92)	0.34 (104)	2.06 (101)	0.34 (103)
Triplet				
2219	1.40 (94)	0.36 (97)	1.46 (98)	0.37 (99)
2221	1.64 (97)	0.24 (101)	1.81 (107)	0.25 (106)
2222	1.92 (97)	0.25 (103)	2.10 (106)	0.26 (108)
Paired strains				
6989	2.92 (101)	0.61 (110)	3.01 (104)	0.57 (103)
3225	3.03 (102)	0.58 (104)	3.06 (103)	0.57 (102)

Figures in parentheses are percentages of the amount of coagulase produced in presence of antiobitics compared to that produced in absence of antibiotics

Table V. The effect of sub -MIC levels of enoxacin on the production of coagulase when related to total cell protein

Strain	1/2 MIC		1/4 MIC	
	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein
Sensitive bacteria				
12009	3.42 (106)	0.79 (99)	3.33 (103)	0.82 (103)
E3T	2.24 (105)	0.33 (103)	2.15 (101)	0.32 (99)
Oxford	1.67 (104)	0.33 (102)	1.58 (98)	0.33 (103)
Cowan 1	2.28 (103)	0.44 (101)	2.14 (97)	0.46 (104)
Wood 46	0.88 (102)	0.22 (100)	0.87 (101)	0.22 (101)
Resistant bacteria				
cip 63	2.85 (99)	0.83 (97)	2.88 (100)	0.88 (102)
cip 86	1.47 (97)	0.33 (104)	1.55 (102)	0.33 (103)
cip 92	1.26 (98)	0.22 (100)	1.34 (104)	0.23 (105)
cip 103	2.59 (98)	0.59 (90)	2.67 (101)	0.67 (102)
cip 132	2.56 (99)	0.41 (94)	2.51 (97)	0.44 (100)
411-87	2.64 (103)	0.71 (106)	2.51 (98)	0.70 (101)
417-87	2.59 (105)	0.64 (104)	2.42 (98)	0.61 (99)
591-89	1.61 (101)	0.34 (115)	1.65 (104)	0.33 (102)
4953-88	2.19 (107)	0.36 (115)	2.11 (103)	0.31 (101)
5538-88	2.76 (101)	0.56 (115)	2.78 (102)	0.50 (103)
8984-88	2.10 (100)	0.30 (91)	2.08 (104)	0.34 (104)
Triplet				
2219	1.52 (102)	0.35 (95)	1.49 (100)	0.39 (105)
2221	1.81 (107)	0.24 (99)	1.67 (99)	0.24 (101)
2222	2.16 (109)	0.24 (101)	1.98 (98)	0.24 (101)
Paired strains				
6989	3.21 (111)	0.55 (100)	2.83 (98)	0.57 (103)
3225	3.15 (106)	0.55 (99)	2.88 (97)	0.57 (102)

Figures in parentheses are percentages of the amount of coagulase produced in presence of antiobitics compared to that produced in absence of antibiotics

Table VI. The effect of sub-MIC levels of methicillin on the production of coagulase when related to total cell protein

Strain	1/2 MIC		1/4 MIC	
	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein
Sensitive strains				
12009	3.59 (111)	0.84 (105)	3.20 (99)	0.82 (102)
E3T	2.36 (111)	0.32 (100)	2.15 (101)	0.33 (104)
Oxford	1.63 (101)	0.33 (103)	1.64 (102)	0.33 (103)
Cowan 1	2.14 (97)	0.44 (99)	2.30 (104)	0.45 (102)
Wood 46	0.84 (98)	0.22 (101)	0.90 (105)	0.22 (101)
Resistant strains				
cip 63	2.97 (103)	0.90 (105)	3.05 (106)	0.87 (101)
cip 86	1.40 (92)	0.35 (108)	1.54 (101)	0.33 (104)
cip 92	1.28 (99)	0.23 (103)	1.32 (102)	0.23 (103)
cip 103	2.61 (100)	0.61 (92)	2.67 (101)	0.65 (99)
cip 132	2.59 (100)	0.44 (100)	2.62 (101)	0.43 (98)
411-87	2.61 (102)	0.71 (106)	2.64 (103)	0.70 (102)
417-87	2.47 (100)	0.62 (100)	2.45 (99)	0.67 (102)
591-89	1.61 (101)	0.37 (115)	1.54 (97)	0.33 (104)
4953-88	2.15 (105)	0.30 (98)	2.07 (101)	0.32 (103)
5538-88	2.92 (107)	0.56 (115)	2.78 (102)	0.49 (101)
8984-88	2.14 (105)	0.34 (103)	2.06 (101)	0.34 (103)
Triplet				
2219	1.55 (104)	0.37 (99)	1.53 (103)	0.38 (104)
2221	1.74 (103)	0.24 (102)	1.79 (106)	0.24 (102)
2222	2.02 (102)	0.24 (102)	2.16 (109)	0.24 (101)
Paired strains				
6989	2.92 (101)	0.57 (104)	3.01 (104)	0.56 (102)
3225	3.09 (104)	0.58 (103)	2.94 (99)	0.57 (101)

Figures in parentheses are percentages of the amount of coagulase produced in presence of antiobitics compared to that produced in absence of antibiotics

Table VII. The effect of sub-MIC levels of gentamicin on the production of coagulase when related to total cell protein

Strain	1/2 MIC		1/4 MIC	
	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein
Sensitive bacteria				
12009	1.91 (51)	0.46 (57)	2.87 (89)	0.79 (99)
E3T	1.28 (60)	0.19 (60)	1.96 (92)	0.32 (100)
Oxford	0.68 (42)	0.15 (47)	1.38 (86)	0.32 (99)
Cowan 1	0.86 (39)	0.20 (45)	1.61 (72)	0.44 (99)
Wood 46	0.45 (52)	0.13 (61)	0.70 (90)	0.22 (100)
Resistant bacteria				
cip 63	1.47 (51)	0.49 (57)	2.88 (100)	0.88 (102)
cip 86	0.65 (43)	0.16 (50)	0.73 (48)	0.36 (114)
cip 92	0.84 (65)	0.16 (71)	1.30 (101)	0.23 (103)
cip 103	1.45 (55)	0.41 (62)	2.30 (87)	0.61 (92)
cip 132	1.27 (49)	0.25 (56)	2.12 (82)	0.48 (103)
411-87	1.59 (62)	0.37 (53)	2.18 (85)	0.63 (92)
417-87	1.01 (41)	0.37 (59)	2.07 (84)	0.62 (100)
591-89	0.83 (52)	0.19 (59)	1.49 (94)	0.34 (106)
4953-88	1.13 (55)	0.19 (62)	2.13 (104)	0.34 (106)
5538-88	1.47 (54)	0.30 (61)	1.94 (71)	0.56 (115)
8984-88	1.08 (53)	0.20 (60)	1.96 (96)	0.34 (103)
Triplet				
2219	0.92 (62)	0.24 (65)	1.36 (91)	0.37 (99)
2221	1.00 (59)	0.12 (50)	1.55 (92)	0.24 (101)
2222	1.17 (59)	0.13 (55)	1.72 (87)	0.24 (102)
Paired strains				
6989	1.24 (43)	0.24 (44)	2.46 (85)	0.57 (104)
3225	1.16 (39)	0.22 (40)	2.49 (84)	0.58 (104)

Figures in parentheses are percentages of the amount of coagulase produced in presence of antiobitics compared to that produced in absence of antibiotics

Table VIII. The effect of sub-MIC levels of chloramphenicol on the production of coagulase when related to total cell protein

Strain	1/2 MIC		1/4 MIC	
	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein
Sensitive bacteria				
12009	1.78 (55)	0.41 (51)	3.13 (97)	0.84 (105)
E3T	1.04 (49)	0.13 (40)	2.00 (94)	0.83 (103)
Oxford	0.60 (37)	0.13 (40)	1.45 (90)	0.32 (99)
Cowan 1	0.77 (35)	0.09 (39)	2.01 (91)	0.44 (101)
Wood 46	0.35 (41)	0.09 (43)	0.77 (90)	0.22 (100)
Resistant bacteria				
cip 63	2.88 (100)	0.79 (92)	2.88 (100)	0.90 (105)
cip 86	0.78 (51)	0.20 (63)	1.23 (81)	0.33 (94)
cip 92	0.71 (55)	0.12 (54)	1.05 (79)	0.22 (90)
cip 103	1.50 (57)	0.42 (63)	2.30 (87)	0.61 (92)
cip 132	1.17 (45)	0.25 (56)	2.38 (92)	0.44 (100)
411-87	1.18 (46)	0.30 (50)	2.64 (103)	0.63 (92)
417-87	1.36 (55)	0.33 (53)	1.78 (72)	0.67 (93)
591-89	0.83 (52)	0.17 (53)	1.19 (75)	0.34 (102)
4953-88	1.11 (54)	0.17 (55)	1.76 (86)	0.36 (105)
5538-88	1.06 (39)	0.20 (41)	2.51 (92)	0.56 (103)
8984-88	0.86 (42)	0.20 (60)	2.06 (101)	0.30 (91)
Triplet				
2219	0.73 (49)	0.93 (55)	1.30 (87)	0.36 (97)
2221	0.93 (55)	0.12 (52)	1.27 (75)	0.24 (91)
2222	0.77 (39)	0.12 (51)	1.60 (81)	0.22 (91)
Paired strains				
6989	1.30 (45)	0.28 (50)	2.51 (87)	0.55 (100)
3225	1.75 (59)	0.31 (55)	2.79 (94)	0.58 (104)

Figures in parentheses are percentages of the amount of coagulase produced in presence of antiobitics compared to that produced in absence of antibiotics

Table IX. The effect of sub-MIC levels of tetracycline on the production of coagulase when related to total cell protein

Strain	1/2 MIC		1/4 MIC	
	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein	Soluble ng coagulase/ mg protein	Bound ng coagulase/ mg protein
Sensitive bacteria				
12009	1.32 (41)	0.42 (52)	2.42 (75)	0.84 (105)
E3T	1.02 (48)	0.20 (61)	1.90 (105)	0.32 (100)
Oxford	0.84 (52)	0.16 (49)	1.48 (92)	0.34 (99)
Cowan 1	0.91 (41)	0.22 (50)	1.86 (84)	0.42 (101)
Wood 46	0.48 (56)	0.12 (55)	0.79 (92)	0.22 (100)
Resistant bacteria				
cip 63	3.05 (106)	0.84 (98)	2.88 (100)	0.83 (97)
cip 86	0.74 (49)	0.14 (45)	1.20 (79)	0.30 (99)
cip 92	0.54 (42)	0.12 (56)	1.12 (87)	0.22 (100)
cip 103	1.35 (51)	0.30 (45)	2.48 (94)	0.61 (92)
cip 132	1.37 (53)	0.22 (50)	2.05 (79)	0.44 (100)
411-87	1.82 (71)	0.38 (55)	2.10 (82)	0.71 (106)
417-87	1.61 (65)	0.33 (53)	2.49 (101)	0.62 (100)
591-89	0.67 (42)	0.15 (48)	1.46 (92)	0.30 (99)
4953-88	1.05 (51)	0.18 (57)	1.70 (85)	0.30 (101)
5538-88	2.92 (107)	0.52 (107)	2.70 (99)	0.46 (99)
8984-88	1.00 (49)	0.18 (55)	1.94 (95)	0.34 (103)
Triplet				
2219	0.82 (55)	0.19 (50)	1.36 (91)	0.37 (102)
2221	0.91 (54)	0.12 (49)	1.47 (87)	0.20 (101)
2222	0.77 (39)	0.10 (40)	1.68 (85)	0.25 (103)
Paired strains				
6989	1.04 (36)	0.24 (43)	2.28 (79)	0.55 (90)
3225	1.25 (42)	0.25 (44)	2.55 (86)	0.50 (95)

Figures in parentheses are percentages of the amount of coagulase produced in presence of antiobitics compared to that produced in absence of antibiotics

Figure X. The effect of sub-MIC levels of ciprofloxacin and enoxacin on the production of α haemolysin when related to total cell protein

Strain	Ciprofloxacin		Enoxacin	
	1/4 MIC HU/mg	1/2 MIC HU/mg	1/4 MIC HU/mg	1/2 MIC HU/mg
Sensitive bacteria				
Wood 46	258.4 (68)	125.0 (52)	247.0 (65)	UD
E3T	6.4 (40)	UD	11.2 (70)	UD
Oxford	12.1 (55)	8.1(30)	19.8 (90)	11.1 (41)
12009	243.0 (90)	UD	162.0 (60)	57.0 (30)
Cowan	9.7 (65)	10.4 (61)	15.2 (101)	11.7 (69)
Resistant bacteria				
cip 63	16.2 (85)	9.1 (61)	9.5 (50)	UD
cip 86	74.2 (70)	72.0 (61)	44.0 (41)	UD
cip 92	213.0 (99)	202.0 (90)	217.2 (101)	199.0 (89)
cip 103	14.0 (70)	4.1 (34)	16.0 (80)	3.7 (31)
cip 132	27.0 (90)	27.6 (52)	27.0 (90)	17.0 (32)
411-87	6.5 (25)	UD	10.4 (40)	UD
417-87	12.1(101)	12.0 (80)	6.6 (55)	UD
591-89	10.4 (40)	UD	27.3 (105)	UD
4953-88	12.8 (80)	4.1 (41)	13.0 (81)	UD
5538-88	7.1 (71)	UD	UD	UD
8984-88	6.2 (41)	UD	15.5 (103)	UD
Triplet				
2219	UD	UD	UD	UD
2221	UD	UD	UD	UD
2222	UD	UD	UD	UD
Paired strains				
6989	218.0 (100)	130.0 (71)	113.4 (52)	73.2 (40)
3225	154.2 (94)	81.0 (52)	147.6 (90)	68.0 (43)

Figures in parentheses are percentages of the amount of α haemolysin produced in presence of antiobitics compared to that produced in absence of antibiotics

Figure XI. The effect of sub-MIC levels of gentamicin and methicillin on the production of α haemolysin when related to total cell protein

Strain	Gentamicin		Methicillin	
	1/4 MIC HU/mg	1/2 MIC HU/mg	1/4 MIC HU/mg	1/2 MIC HU/mg
Sensitive bacteria				
Wood 46	361.0 (95)	222.0 (92)	1050 (>200)	2080 (>200)
E3T	15.2 (95)	7.5 (94)	600 (>200)	35 (>200)
Oxford	13.5 (90)	24.0 (88)	55 (>200)	182 (>200)
12009	267.0 (99)	192.0 (101)	60 (>200)	531 (>200)
Cowan 1	20.0 (91)	13.1 (77)	100 (>200)	15 (>200)
Resistant bacteria				
cip 63	18.2 (96)	14.4 (96)	191 (>200)	473 (>200)
cip 86	105 (99)	117.0 (99)	405 (>200)	292 (>200)
cip 92	204.0 (95)	207.0 (92)	390 (>200)	896 (>200)
cip 103	18.4 (92)	10.4 (87)	150 (>200)	149 (>200)
cip 132	27.6 (92)	44.5 (84)	350 (>200)	597 (>200)
411-87	20.8 (80)	29.0 (72)	410 (>200)	339 (>200)
417-87	11.0 (92)	14.1 (94)	110 (>200)	1137 (>200)
591-89	25.7 (99)	25.5 (92)	130 (>200)	133 (>200)
4953-88	13.4 (84)	8.2 (82)	350 (>200)	62 (>200)
5538-88	9.4 (94)	10.9 (91)	410 (>200)	113 (>200)
8984-88	13.5 (96)	11.3 (87)	210 (>200)	106 (>200)
Triplet				
2219	UD	UD	UD	UD
2221	UD	UD	UD	UD
2222	UD	UD	UD	UD
Paired strains				
6989	198.4 (91)	172.0 (41)	740 (>200)	196 (>200)
3225	146.0 (89)	143.0 (92)	350 (>200)	57 (>200)

Figures in parentheses are percentages of the amount of α haemolysin produced in presence of antiobitics compared to that produced in absence of antibiotics

Figure XII. The effect of sub-MIC levels of chloramphenicol and tetracycline on the production of α haemolysin when related to total cell protein

Strain	Chloramphenicol		Tetracycline	
	1/4 MIC HU/mg	1/2 MIC HU/mg	1/4 MIC HU/mg	1/2 MIC HU/mg
Sensitive bacteria				
Wood 46	80.0 (21)	UD	156.0 (41)	UD
E3T	12.8 (80)	UD	4.5 (30)	2.0 (25)
Oxford	21.8 (99)	13.0 (49)	16.0 (71)	13.0 (47)
12009	162.0 (60)	99.0 (52)	248.0 (92)	116.0 (61)
Cowan 1	UD	UD	UD	UD
Resistant bacteria				
cip 63	13.3 (70)	10.0 (67)	9.8 (52)	UD
cip 86	97.5 (92)	UD	33.0 (31)	UD
cip 92	213.0 (99)	47.0 (21)	144.0 (67)	UD
cip 103	15.0 (75)	UD	10.2 (51)	UD
cip 132	15.3 (51)	33.0 (62)	21.6 (72)	2.4 (49)
411-87	UD	UD	26.3 (101)	39.0 (101)
417-87	12.1(101)	13.6 (91)	UD	UD
591-89	15.9 (61)	UD	25.7 (99)	UD
4953-88	10.3 (103)	UD	UD	UD
5538-88	UD	UD	UD	UD
8984-88	6.7 (45)	UD	6.3 (42)	UD
Triplet				
2219	UD	UD	UD	UD
2221	UD	UD	UD	UD
2222	UD	UD	UD	UD
Paired strains				
6989	178.8 (82)	75.0 (41)	200.0 (92)	46.0 (25)
3225	162.4 (99)	139.0 (89)	129.6 (79)	66.0 (42)

Figures in parentheses are percentages of the amount of α haemolysin produced in presence of antiobitics compared to that produced in absence of antibiotics

Figure XIII. The effect of sub-MIC levels of ciprofloxacin and enoxacin on the production of δ haemolysin when related to total cell protein

Strain	Ciprofloxacin		Enoxacin	
	1/4 MIC HU/mg	1/2 MIC HU /mg	1/4 MIC HU/mg	1/2 MIC HU /mg
Sensitive bacteria				
E5662	100 (99)	92.0 (91)	85.9 (85)	79.8 (79)
E3T	72.9 (90)	57.5 (71)	68.0 (84)	48.6 (60)
Oxford	2.3 (90)	2.0 (80)	1.7 (69)	UD
12009	18.0 (90)	16.4 (82)	19.8 (99)	9.8 (49)
Cowan 1	0.4 (80)	0.3 (60)	0.3 (60)	UD
Wood 46	11.9 (79)	9.8 (65)	14.3 (95)	13.5 (90)
Resistant bacteria				
cip 63	26.0 (40)	UD	35.8 (55)	UD
cip 86	36.5 (89)	25.4 (62)	39.0 (95)	26.7 (65)
cip 92	47.8 (81)	25.4 (43)	41.9 (71)	23.0 (39)
cip 103	0.8 (91)	0.7 (77)	0.9 (99)	0.5 (55)
cip 132	9.8 (89)	7.2 (65)	10.7 (97)	4.4 (40)
411-87	1.7 (80)	1.5 (72)	2.1 (101)	1.9 (90)
417-87	16.2 (90)	UD	16.2 (90)	14.4 (80)
591-89	UD	UD	UD	UD
4953-88	UD	UD	UD	UD
5538-88	0.2 (50)	UD	UD	UD
8984-88	4.0 (99)	2.1 (52)	3.6 (90)	2.9 (72)
Triplet				
2219	UD	UD	UD	UD
2221	UD	UD	UD	UD
2222	UD	UD	UD	UD
Paired strains				
6989	31.9 (91)	28.0 (80)	19.3 (55)	UD
3225	22.5 (75)	UD	31.2 (104)	18.0 (60)

Figures in parentheses are percentages of the amount of δ haemolysin produced in the presence of antibiotics compared to that produced in the absence

Figure XIV. The effect of sub-MIC levels of gentamicin and methicillin on the production of δ haemolysin when related to total cell protein

Strain	Gentamicin		Methicillin	
	1/4 MIC HU/mg	1/2 MIC HU /mg	1/4 MIC HU/mg	1/2 MIC HU /mg
Sensitive bacteria				
E5662	105.0 (104)	100.0 (99)	239 (>200)	250 (>200)
E3T	78.6 (97)	77.8 (96)	289 (>200)	360 (>200)
Oxford	2.5 (98)	2.3 (90)	15 (>200)	10 (>200
12009	20.4 (102)	20.2 (101)	35 (>200)	40 (>200)
Cowan 1	0.5 (94)	0.5 (92)	4 (>200)	4 (>200)
Wood 46	15.3 (102)	13.5 (90)	70 (>200)	90 (>200)
Resistant bacteria				
cip 63	61.1 (94)	61.1 (94)	200 (>200)	400 (>200)
cip 86	40.6 (99)	39.8 (97)	100 (>200)	150 (>200)
cip 92	58.4 (99)	59.6 (101)	79 (>200)	200 (>200)
cip 103	0.9 (98)	0.9 (101)	2 (>200)	2 (>200)
cip 132	11.1 (101)	10.9 (99)	30 (>200)	40 (>200)
411-87	2.0 (94)	1.8 (84)	6 (>200)	6 (>200)
417-87	17.6 (98)	17.8 (99)	40 (>200)	60 (>200)
591-89	UD	UD	UD	UD
4953-88	UD	UD	UD	UD
5538-88	0.4 (104)	0.4 (101)	1 (>200)	2 (>200)
8984-88	4.0 (99)	3.9 (97)	9 (>200)	12 (>200)
Triplet				
2219	UD	UD	UD	UD
2221	UD	UD	UD	UD
2222	UD	UD	UD	UD
Paired strains				
6989	33.6 (96)	31.9 (91)	70 (>200)	86 (>200)
3225	29.4 (98)	29.1 (97)	62 (>200)	60 (>200)

Figures in parentheses are percentages of the amount of δ haemolysin produced in the presence of antibiotics compared to that produced in the absence

Figure XV. The effect of sub-MIC levels of chloramphenicol and tetracycline on the production of δ haemolysin when related to total cell protein

Strain	Chloramphenicol		Tetracycline	
	1/4 MIC HU/mg	1/2 MIC HU /mg	1/4 MIC HU/mg	1/2 MIC HU /mg
Sensitive bacteria				
E5662	49.5 (49)	UD	51.5 (49)	UD
E3T	82.6 (102)	81.8 (101)	40.5 (50)	0.8 (30)
Oxford	1.5 (61)	1.0 (40)	1.8 (70)	UD
12009	16.0 (80)	6.2 (31)	14.2 (71)	UD
Cowan 1	0.2 (42)	UD	0.4 (70)	UD
Wood 46	7.1 (47)	4.5 (30)	9.3 (62)	UD
Resistant bacteria				
cip 63	6.5 (10)	UD	13.7 (21)	UD
cip 86	24.6 (60)	16.8 (41)	30.8 (75)	17.2 (42)
cip 92	24.2 (41)	UD	23.6 (40)	UD
cip 103	0.9 (102)	0.6 (70)	0.5 (57)	UD
cip 132	11.1 (101)	9.8 (89)	3.5 (32)	UD
411-87	1.3 (60)	UD	0.8 (40)	UD
417-87	16.4 (91)	14.6 (81)	16.6 (92)	UD
591-89	UD	UD	UD	UD
4953-88	UD	UD	UD	UD
5538-88	0.4 (104)	0.2 (41)	UD	UD
8984-88	4.0 (99)	UD	3.6 (89)	2.8 (70)
Triplet				
2219	UD	UD	UD	UD
2221	UD	UD	UD	UD
2222	UD	UD	UD	UD
Paired strains				
6989	14.0 (40)	UD	15.1 (43)	UD
3225	18.3 (61)	UD	11.1 (37)	UD

Figures in parentheses are percentages of the amount of δ haemolysin produced in the presence of antibiotics compared to that produced in the absence